

Why not reform the United Nations?

The low-key celebrations of the half-century of the United Nations has served chiefly as a reminder that the organization is ripe for reform — but not abolition.

THE United Nations celebrated its fiftieth anniversary last weekend with modesty that was all too appropriate, but nevertheless a disappointment. The UN Charter is the product of negotiations carried out between the major powers when the Second World War still had many battles to run. That so many could have been so optimistic that the problems ahead of them would prove tractable is an exhilarating proof that hope can sometimes triumph over despair. The disappointment in last week's proceedings is that the United Nations should now be as dispirited as appears to be the case, and just when it seemed that it was coming into its own. Whatever happened to the "new world order" President George Bush was talking of just a few years ago?

Part of the truth is that organizations like the United Nations can expect to be popular among their constituents only sporadically, when by accident they have managed to please everybody. For most of the time, they must expect a majority of malcontents among their members. (The European Union is learning as much the hard way.) The United Nations also suffers from perennial budget problems, which are dispiriting and are inevitably worsened by present efforts to keep peacekeepers in a dozen potential (and actual) battlefields. Dr Boutros Boutros Ghali, the director-general, is right to complain that it makes no sense for the Security Council to will a peacekeeping force here or there when its members will not meet the cost. The United Nations is not to blame for all its own ills.

Yet the present clamour for reform (especially in the United States) cannot be dismissed as unavoidable discontent. Indeed, it is no surprise that an organization able just to hold itself together when the Cold War severely limited its capacity to act is found wanting now that circumstances have given it a wider canvas. The most obvious present difficulty is the diversity of the functions member states expect of the United Nations. At one end of the spectrum is the general obligation to assist the development of developing countries; at the height of the Cold War, there was nothing much else to do. At the other are military and quasi-military activities. (The Korean and Gulf Wars are in the first category, the intervention in Bosnia in the second.) In between are the activities of the specialized agencies (the World Health Organization, WHO, for example) and the diplomatic task of negotiating international treaties.

How to make that mess manageable? An obvious need is to give the specialized agencies more autonomy and more responsibility. Their management now falls awkwardly

between the United Nations in New York and their own general assemblies, in which civil servants are the chief participants. Their heads are appointed on political grounds ("Who's turn is next?"); if they play their cards shrewdly, they can do a poor job and still be elected for a second term — WHO is the latest illustration of that principle. The bureaucracy generally has the whip-hand over the meritocracy. The best solution is that the directors of these organizations, who must be in charge of their own affairs from day to day, should be responsible to a body of identifiable trustees in general, and to the corps of international civil servants month by month. That way, there would be more action and less spending.

At the other extreme, similar expedients would make sense. Boutros Boutros Ghali is not qualified as a general, but has lately been functioning as a kind of commander-in-chief. He has military advisers, but they come and go and tend to be superannuated. It may at this stage be too much to ask that the United Nations should recruit military forces of its own, but there is a case for a peacekeeping staff college and for a small hard core of military people on the staff. □

Clinton two strikes down

The US president has lost his nomination for surgeon-general and made a mess of an immunization programme.

THAT hardly anything, these days, goes right for President Bill Clinton is no surprise: both houses of the US Congress are dominated by his political opponents. Thus the Senate's failure last week to approve the nomination of Dr Henry W. Foster as surgeon-general, but to leave it (and him) in indefinite suspense, was predictable. The decisions not to end debate on the question, but also not to bring the issue to a vote, offers the president a distasteful choice: either nominate somebody else or make do without a surgeon-general.

The president is right to complain that the Senate's actions were politically motivated, but politics are rarely distant from nominations procedures. On this occasion, the Republican leader in the Senate, Mr Robert Dole, who is at present the leading opposition candidate for Clinton's own job in the 1996 election, made three telling points. He demonstrated to Clinton the power of the new Senate, he bested one of his declared rivals for the presidency (Senator Phil Gramm) by showing that he could scupper the nomina-



tion by more subtle means than Gramm's proposed filibuster and he will also have won friends among the anti-abortion lobbyists by doing down Foster, who is a gynaecologist who has "confessed" to having carried out 37 abortions during his professional career. The injustice is literal: abortion, subject to medical investigation and approval, is still legal in the United States. Many of Clinton's early nominations were lost because those concerned had acted illegally in, for example, failing to pay taxes in respect of domestic employees, but it is cruel to lose a good nominee on the grounds that he discharged professional responsibilities within the law.

But on another disconcerting issue, Clinton must shoulder the blame more directly. At the outset of his presidency, he launched a programme called "Vaccines for Children" with the admirable goal of providing, free of charge, vaccines against the common infectious diseases of infancy. It is true that the initial rhetoric rang false; Clinton's claim that vaccine manufacturers were pursuing "profits at the expense of our children" overlooked the well-documented retreat of US pharmaceutical manufacturers from the business, to a large extent because of the high costs of legal liability suits (or of insurance against them). Nevertheless, the Congress approved the programme in 1993, believing that some 60 per cent of children in the United States would be eligible for free vaccines.

Now the General Accounting Office, an independent office working for the Congress, has produced a disturbing report on the administration of the programme. Briefly, it appears to be a hopeless muddle. Those in charge have made the mistakes that used to be common in developing countries, and have supposed that the mere existence of stocks of vaccines would ensure the immunization of children. Too little has been done to persuade parents of the benefits of vaccination, record-keeping has ranged from the non-existent to the unreliable and there are doubts that the eligibility criteria have been kept. This mess is a gift to Clinton's political opponents. Clinton would be well advised to mount a pre-emptive reform before the Republicans kill the programme altogether. Foster could tell him what to do. □

Brent Spar, broken spur

Shell Oil's decision not to sink a used oil-rig at sea is a needless dereliction of rationality.

POSTERITY will bestow a strange honour on Britain's Prime Minister, Mr John Major. Last week, which is that in which he announced his resignation as leader of the government Conservative Party in order to seek re-election to that post, he also offered the House of Commons a sterling defence of the decision by the Shell Oil Company to scupper the Brent Spar, a defunct oil storage platform, in the deep Atlantic Ocean. Thus was a gambler's desperate throw juxtaposed with a piece of solid rationality. But Major earned no kudos for his courage. Shell said that it would cave in to environmental pressure almost immediately after Major had fin-

ished speaking, and would arrange for the platform to be dismantled on land instead. An embattled prime minister deserves better luck than that.

Quite why Shell changed its mind is far from clear. The environmental group Greenpeace, with 500,000 members in Britain, will be eager to claim the credit. Greenpeace activists occupied the platform while it was still in place and, afterwards, when it was being towed to its last resting place. The attention paid to those events inevitably resulted in a wave of protest across Europe. The German government, which has an interest in keeping its domestic Green Party out of the arms of the Social Democrats, came out against Shell's plan even to the extent of raising the issue at the G7 summit at Halifax two weeks ago. No doubt it has also been using its influence behind the scenes in ways calculated to discomfit a multinational company such as Shell.

The irony in this episode, which has embarrassed Major, has done no credit to Shell and has again exposed the shallowness of Greenpeace's arguments on scientific issues, is that the environmental effect on deep-sea life of dumping the Brent Spar would be minimal or even beneficial. The issue, of course, is not the dumping of the platform itself, but of its contents: a substantial dollop of sludge containing 40 tonnes of oil and hundreds of kilograms of toxic heavy metals. The campaigners have been claiming that it would be irresponsible to dump this brew on the deep ocean floor, but Nisbet and Fowler suggest on page 715 of this issue that there is a different tale to tell.

Nobody suggests that the ocean floor is potentially a garbage dump of infinite capacity or that it is a barren wasteland fit for no other use: the ecology of the deep sea is as rich and varied as anything on land, and different in many fundamental ways. But, far from finding heavy-metal residues lethal or even mildly unappetizing, the bacteria of the ocean floor would have greeted the arrival of Brent Spar as if all their Christmases had come at once. Many deep-sea microbes require heavy metals as electron or energy sources in their metabolism. Ocean-floor organisms use heavy metals, hydrocarbons and brimstone because they have become adapted to quantities of these materials surging from hydrothermal vents. As Nisbet and Fowler show as an example, the Broken Spur vent field in the North Atlantic churns out up to five million tonnes of heavy metals annually, by which yardstick Brent Spar's cargo is insignificant.

What happens now? Shell will have the platform dismantled on land instead of dumping it, which must be a more hazardous process that will eventually cost the company's customers a small fortune. Greenpeace will boast of its pyrrhic victory (and its membership will no doubt increase). And, helped by the pusillanimity of governments like the German, public confusion about the circumstances in which ocean dumping can be considered safe will be increased. What matters is that the deep ocean floor is very different from the continental shelf: the problems of pollution and over-fishing in inshore waters, such as the North Sea, are very real. It is in shallow seas that human activity (and restraint) can make a difference. That is where public attention (and that of campaigners) should be directed. □

US energy department backs down on plan for A-bomb victim studies

Washington and Tokyo. The US Department of Energy has reversed its controversial plan to strip the National Academy of Sciences (NAS) of responsibility for managing Radiation Effects Research Foundation (RERF) in Japan, which carries out long-term studies of the effects of the atomic bombs dropped on Japan during the Second World War.

RERF scientists had reacted with fury to a letter from the DoE letter ascribing its plan to the alleged secrecy of RERF's work. In response, the DoE said on Monday that the academy contract to manage RERF would be extended for two years, while an "international committee of distinguished scientists" assesses RERF's work.

Earlier this year, the DoE caused uproar among radiation scientists in Japan and the United States by announcing plans to transfer the academy's responsibility for the US side of the RERF, which is based in Hiroshima and jointly financed by Japan and the United States, to a US university or group of universities (see *Nature* 374, 490; 1995).

This month, Hazel O'Leary, the US energy secretary, said that she would drop the academy as manager of the programme, a role which it has held since 1947, claiming that this was being done because of the ending of the Cold War and her desire to move away from secret research on the biological effects of radiation.

"The need for secret research on the biological effects of radiation has ended," she wrote to Bruce Alberts, president of the academy, on 14 June. "The worldwide interest in radiation health issues has grown beyond the concerns that provoked the armed services to ask [US President] Truman to appoint the academy in 1947."

But Tara O'Toole, assistant secretary for environment, safety and health, spoke with Alberts this week and agreed to extend the academy's contract for at least two years. Steven Galson, chief medical officer at the energy department, said the abrupt reversal followed "an incredible outpouring of concern about the decision to go with a university contractor".

In a statement suggesting that DoE's vacillation on the issue may reflect sharp divisions of opinion within the department, Galson added that the O'Leary letter was "not trying to imply that [the academy] and RERF were doing secret research".

US and Japanese researchers at RERF in Hiroshima are angry at the suggestion that their research is secret. "RERF does not engage in secret research," say Dale Preston, head of RERF's department of statistics, and Kiyohiko Mabuchi, head of the

department of epidemiology.

They say that the foundation's work "has been and continues to be more public than any other study of radiation effects". They add that "this interpretation illustrates DoE's continued misunderstanding of the nature of the work at RERF, and [the academy's] role in these studies".

Before the policy reversal, one energy department official said that the RERF had never shaken off the influence of its origins as the Atomic Bomb Casualty Commission (ABCC), which, he pointed out, was established primarily to provide the US atomic bomb programme with secret information on the effects of exposure to radiation.

The official said that the early mission of the ABCC had been "absolutely without question" to perform secret research to benefit the weapons programme. Despite opening up later — especially after 1975, when the commission accepted Japanese funding and became RERF — he said it had remained "isolated from the mainstream of public health research".

He cited the experience of Alice Stewart, a prominent, if controversial, British cancer expert, who has long challenged various official conclusions about the impact of exposure to radiation, and who he said had been denied "open and free access" to RERF data during the 1980s.

O'Leary is engaged in a highly public campaign to disentangle the Department of Energy and other US government agencies from secret radiation experiments, both past and present. The campaign has brought her department into sharp conflict with other agencies, including the Department of

Defense and the National Institutes of Health.

Itsuzo Shigematsu, chairman of RERF, says he is "surprised" by O'Leary's letter, and speculates that her view is based on events surrounding the early days of the ABCC. He also says that it is "completely wrong" for DoE officials to suggest that RERF has denied Stewart access to data.

But Preston acknowledges that some raw data was withheld from Stewart, claiming that this was on the grounds that sharing it "would violate the privacy of victims." Nevertheless, he claims that RERF has recently been "very responsive" to Stewart and other outside researchers, and describes charges that the foundation has been isolated from other research efforts as "outrageous". Preston suggests that the real motivation for the proposed change in management responsibility is the DoE's desire to save money.

O'Leary's comments were sent in response to a letter of 28 April from Alberts forwarding a resolution passed by the academy in protest at DoE's plans, and referring to widespread objections from the scientific community in Japan and the United States to the planned reorganization.

In April, the RERF Science Council, a binational group of scientists responsible for the foundation's scientific programme, also expressed concern about the plans. It called on the department to defer any decision on restructuring until the foundation's activities have been reviewed by an external team of experts. The Department of Energy now says it accepts the council's recommendation for such a review.

Colin Macilwain & David Swinbanks

French physicists attack bomb tests

London. French scientists have mounted a series of protests against their government's decision to resume nuclear testing in the South Pacific atoll of Mururoa after a four-year suspension.

Last week, the National Union of Scientists issued a public statement condemning the decision, and numerous petitions are being circulated in the scientific community demanding that the underground tests should not be resumed.

The French Academy of Sciences, many of whose members have supported the protests as individuals, also debated the issue earlier this week, although by the end of the meeting no decision had been reached on whether to issue a formal

declaration on the resumption of tests.

France has been testing nuclear weapons at Mururoa since 1966, with tests being carried out underground since 1974. The tests were suspended in 1992, but the new French President, Jacques Chirac, has now announced that eight further tests will be ►

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Feeling blue: scientists regret fresh testing at Mururoa.

► carried out between September 1995 and May 1996.

Jacques Bouchard, director of military applications at the French Atomic Energy Commission, denies the claims of critics that the purpose of the tests is to develop better weapons, claiming that they are necessary to ensure that present weapons are functional and can be satisfactorily replaced.

"Nuclear weapons last only about 20 years and must be renewed as they age," he says. "This is not a question of improving performance or miniaturization; the weapons in question are more or less the same models as those of a few years ago."

Bouchard also says that at the time of the 1992 moratorium, France had not collected sufficient data to design good computer simulations of tests, and that the resumed testing shows France's commitment to a future complete test ban by allowing the completion of such simulations.

Meanwhile, the impact of the tests remains controversial. Critics such as Bruno Barillot, of the Centre of Documentation and research on Peace and Conflicts in Lyons, claim there is evidence of increased rates of cancer in the region after nuclear testing. (Government officials have responded to such suggestions by arguing that the cancer rates are now in line with those of industrialized countries.)

A report published in 1987 on the effects of underground testing on the atoll concluded that the risks of radioactive pollution in the short term were "negligible". But it added that the longer-term risks were more difficult to evaluate.

Despite the lack of any conclusive evidence of the risks, many scientists have spoken out against the decision to resume testing. They include at least one physicist — Michel Cribier — working at the Atomic Energy Commission, and several individual members of both the academy and the National Ethics Committee.

Critics point out that former President François Mitterrand used expert reports similar to those delivered to Chirac as grounds for not continuing testing. They say that this shows that the decision to resume testing was taken for political, and not scientific or technical, reasons.

One petition being circulated by Pierre Jaeglé and Alain Sureau of the University of Orsay acknowledges that experimentation is needed to reinforce simulation. But it says that this is a cyclical argument that can only be stopped by a political decision.

In addition to ethical and environmental arguments, some scientists say they are also concerned that the large sums of money necessary for the nuclear tests may lead to further reductions in the already limited funding for research for peaceful purposes.

Marianne Grunberg-Manago, the president of the academy, has also expressed disappointment that the academy was not consulted before the decision to resume testing was made.

Kimberly Carr

Balkan physics cruise shifts to dry land after protests

Munich. A high energy physics conference bringing together researchers from countries in the Balkan region has been taking place this week in the northeastern Italian town of Trieste, close to the border with the former Yugoslavia.

But earlier plans to hold the meeting on 'neutral' territory represented by a boat sailing around the Balkan area were abandoned after objections from some potential participants that this would have appeared insensitive to the political context in which it was taking place.

The so-called Four Seas conference still takes its name from the original plan, which was to hold it on board a ship cruising from Trieste to Burgas in Bulgaria across four seas — the Adriatic, the Aegean, the Marmara and the Black Seas.

The meeting has been organized by Georges Charpak, formerly of the European Laboratory for Particle Physics (CERN), and winner of the 1992 Nobel prize for physics. He says that the idea of the cruise was to provide a neutral venue which speakers could attend without compromising their national or political affiliations.

Before the violence that has accompanied the break-up of Yugoslavia, international summer schools in particle physics used to take place regularly in various Yugoslav cities, such as Sarajevo and Dubrovnik. Charpak says he wants the Four Seas conference to revive this tradition, which provided an important opportunity for contact between scientists in the various Balkan countries.

His original, somewhat idealistic, plan was for the cruise to make regular stops to pick up participants from ports in their own countries, in order to reduce the difficulties they might have otherwise experienced in travelling between countries in the region.

But some members of the conference's advisory committee objected to the plan. They claimed that the cruise could have been interpreted as a pleasure trip down the Adriatic coast, giving the impression that the physicists were insensitive to the conflicts raging on land.

The European Commission had already agreed to contribute up to ECU120,000 (US\$158,000) towards the conference. But Charpak, who reluctantly agreed last September to hold the conference on dry

(and non-Balkan) land, says that additional sponsors, such as France's Centre d'Etudes Nucléaires at Saclay near Paris, could have been offended.

Even after the switch to Trieste, however, where the conference is being held at the International School for Advanced Studies, the organizers met substantial resistance to the concept that the meeting could be attended by any scientist in the region, regardless of country of origin.

Many potential participants refused to attend because they did not want to share a platform with a scientist from an enemy country. Despite his own war-time experiences as a member of the French resistance, Charpak says he had not expected to encounter such a violent reaction. "Even physicists not directly involved in the war hate each others' guts", he says.

Charpak says that some physicists from states in the former Yugoslavia refused to attend because they objected to the presence of a speaker from CERN whose father had played a role in the war that they found unacceptable. "It is a kind of craziness," he says. "Some physicists are already ideologically overtaken by the war."

Despite the difficulties, the conference has managed to attract 180 participants, more than half from the Balkan countries, which include Greece and Turkey, as well as several former communist states, such as Albania and Bulgaria. But Charpak says that the numbers from former Yugoslavian countries have been disappointingly low.

Speakers at the meeting included the Nobel prizewinner Carlo Rubbia. Charpak says that the Four Seas conference is planned to become an annual event — and that he eventually hopes that it will be able to take place on the four seas as originally planned.

Alison Abbott

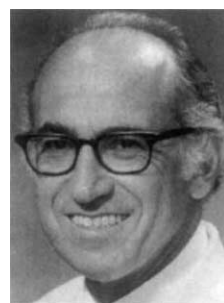
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**Charpak: objections
sunk his cruise plans.**

CERN Photo

Polio pioneer Salk dies

San Diego. **Jonas Edward Salk (right), who developed the first vaccine against poliomyelitis and founded the scientifically and architecturally renowned Salk Institute in La Jolla, died on 23 June aged 80.**



"We are extraordinarily saddened by the loss," said Francis Crick, Nobel prize-winner laureate and president of the Institute. "Few have made one discovery that has benefited humanity so greatly." □

Japan pledges new aid to Minamata victims

Tokyo. After decades of foot-dragging, the Japanese government has at last come forward with a scheme to compensate thousands of victims who were poisoned with mercury-contaminated seafood in the 1950s and 1960s in one of the world's worst cases of industrial pollution.

Last week, the country's three ruling coalition parties — the Liberal Democratic Party, the Social Democratic Party of Japan and the New Party Sakigake — mapped out a plan that will loosen the government's criteria for designating sufferers of so-called Minamata disease.

The plan will also provide financial assistance to the company responsible for the pollution, Chisso Corporation, to help it pay the huge amount of compensation — estimated to be at least ¥25 billion (US\$300 million) — for newly acknowledged victims, many of whom are already dead.

But the government has not accepted any legal responsibility for the disaster. Indeed, its lengthy court battles with Minamata victims have highlighted the lack of redress under Japan's legal system for victims of disasters in which the government has played a role.

Minamata disease is a crippling disorder of the central nervous system that causes victims to lose control of their arms, legs, speech and eyesight. The first such case was recognized in 1956 in the small fishing village of Minamata in southern Japan, and as early as 1957, researchers at the nearby Kumamoto University had linked the disease to traces of mercury found in the brains of dead victims.

But these conclusions were ignored by both the central and local governments. It was not until 11 years later that the government ordered Chisso to stop dumping mercury-laden sludge into Minamata Bay. By that time, thousands of people throughout Japan's southern island of Kyushu had been poisoned with mercury-contaminated seafood from the Minamata area.

The government and Chisso Corporation have recognized about 3,000 severely affected victims, who have each been paid large amounts of compensation. But about 10,000 people with milder symptoms have not been recognized as victims, and many of these have been fighting the government in the courts for recognition and compensation.

There have been six district court rulings over the past decades, three of which have held the government responsible. But the government has appealed against each decision, and, as a result of the snail's pace at which such cases are treated by Japan's legal system, decisions at a higher court level have yet to be made.

Under the old criteria for acknowledging

victims of Minamata disease, these were only officially accepted as such by the government if they suffered severe disabilities such as dysfunction of the motor nerves and narrowed vision. By contrast, under the coalition government's scheme adopted last

week, victims will now be recognized if they suffer any form of paralysis of the central nervous system that can be attributed to mercury poisoning, and if they live (or lived) in the contaminated area.

The government's decision to relax the criteria has resulted from the reduced influence of the Liberal Democratic Party (LDP), which ruled Japan continuously for 38 years until 1993. The LDP and the Environment Agency, which has jurisdiction over the case, steadfastly refused to recognize more victims. But the two other parties in the new coalition government, which are keen to resolve the case, managed to persuade the LDP to make concessions.

The Minamata case is by no means exceptional. The day after the prime minister, Tomiichi Murayama, announced the coalition's Minamata plan, the Supreme Court absolved the government of all blame

in a 20-year court battle over another medical disaster involving the drug chloroquine.

Introduced to Japan in 1955, initially as an antimalaria drug, chloroquine is believed to have caused impaired vision in about 1,300 patients. It was widely used in Japan throughout the 1960s for treatment of rheumatism, epilepsy and kidney inflammation, even though a British medical journal reported in 1959 that the drug is able to cause damage to the retina.

In 1965, the head of the Ministry of Health and Welfare's pharmaceutical department stopped taking the drug for his own rheumatism after learning of its potential side-effects. But the ministry did not order manufacturers to include a warning about possible side-effects until 1969, and did not acknowledge that the severity of the side-effects outweighed its effectiveness in treating kidney inflammation until 1976.

Between 1975 and 1978, many patients with impaired vision sought redress for their injury in the courts. In 1982, the Tokyo District court ruled in their favour and ordered the government, pharmaceutical companies and hospitals to pay ¥3.6 billion in compensation.

This decision was, however, overruled in 1988 by the Tokyo High Court, which absolved the government of blame, and said that only the pharmaceutical companies should pay the compensation — a ruling confirmed by the Supreme Court last week.

Critics of this latest ruling include Gunshiro Yokozawa, who heads a group of chloroquine victims. "As one of the victims, I feel deep anger," he is reported to have said. "This judgement proves that the judicial system is on the side of the government".

David Swinbanks

Technology office heads for reprieve

Washington. The US Office of Technology Assessment (OTA), the congressional body that provides advice to legislators on science and technology issues, won an unexpected reprieve last week from the House of Representatives.

An amendment put forward by Amo Houghton (Republican, New York) to an appropriations bill designed to save the agency but cut its budget from \$22 million to \$15 million and merge it into the larger Congressional Research Office was passed in a surprise vote by 220 to 204.

The result was greeted with jubilation at the OTA, where many staff had been expecting the office to close after appropriations subcommittees in both House and Senate had said that they wanted to elimi-

nate it (see *Nature* 373, 270; 1995).

Indeed, Ron Packard (Republican, California), chairman of the House subcommittee, said after the vote that he would still try to have the OTA's whole budget eliminated when the Senate and House appropriations bills are reconciled in conference.

But the House vote now makes it likely that the OTA will also win support in the Senate, where its work is better known, and only a few Republican defections are needed to win a majority for any move to keep it alive. In the House, 48 Republicans joined Democrats in supporting the OTA, convinced by the argument that Congress needs the office to help it legislate sensibly on complex scientific and technological issues.

Colin Macilwain

Chris Steele-Perkins/MAGNUM

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Lingering legacy: wider criteria will aid more sufferers.

'Faster, cheaper, better' under fire at NASA

Washington. The new Discovery line of low-budget planetary missions being developed by the US National Aeronautics and Space Administration (NASA) is more concerned with economy than with scientific quality. That at least was the verdict of scientists at a recent workshop convened by the agency to review the four-year-old programme.

William Boynton of the University of Arizona, who chaired one of the workshop sessions, said that NASA's only criteria in this year's selection of Discovery missions appear to have been "cost, cost, and cost".

Proposers who designed more complex missions, thinking that they would qualify for funding if they stayed within the \$150-million limit for Discovery projects, were therefore at a disadvantage. So even though hopes were originally high for such projects, "much of the science community is now soured on the programme", said Boynton.

Christopher Russell, for example, of the University of California at Los Angeles, who like Boynton lost out in a recent round of project selections, says that many planetary scientists are now dissatisfied with the Discovery selection process. "The feeling perhaps runs a little deeper than just

having lost a good race," says Russell.

NASA officials deny that the winning missions were chosen on the basis of cost alone. But they concede that many scientists appear to have been confused by the ground rules. "The game needs to be a little clearer," admits Mark Saunders, the Discovery programme manager.

The only concept chosen last March for full funding from among 28 proposals was a \$59-million lunar orbiter mission (see *Nature* 374, 107; 1995). This was also the cheapest proposal. Three more proposals — for a cometary dust sample return, a solar wind sample return and a spacecraft that would send multiple probes into the atmosphere of Venus — were selected for further study; all three came in well under the \$150-million price cap.

Those attending the workshop applauded NASA for holding the two-day event. But they were not shy about pointing out problems with the programme. One common complaint was that preparing Discovery proposals is too expensive.

By requiring extensive details on factors such as mission cost, NASA is leading scientists to spend an average of half a million dollars on each proposal. "The community cannot afford the expense required to prepare Discovery proposals at this level on a continuing basis," said Russell.

NASA officials agreed that some kind of two-step process — with less detail in the preliminary round — could probably be introduced for the next round of proposals, scheduled for late this year or early 1996.

Scientists at the workshop also complained that the weighting factors used for selecting missions were unclear. NASA had explicitly asked proposers to include educational components, community out-

reach, technology transfer and innovative technology in their mission designs. But many believe that these factors were either not considered, or became disadvantages.

According to Russell, reliance on untried new technology, for example, appeared to be a 'net negative' as it increased a mission's risk of failure. Despite the agency's stated desire to encourage innovation, "the use of old technology was rewarded," he said.

The critics charge that NASA misled the community about how much money would be allocated for any one mission. Many planetary scientists were dismayed last year when the agency appeared to lower the unofficial Discovery cost cap to \$100 million, while keeping the official cap at \$150 million (see *Nature*, 369, 594; 1994).

Several formulae were proposed at the workshop for quantifying 'science per dollar'. But there was no consensus as to how small, simple missions could compete on an equal footing with complex, expensive ones. As a result, one suggestion was that Discovery missions should be divided into small, medium and large categories, with each competing separately.

Daniel Goldin, NASA's administrator, appears to be responsible for some of the uncertainty over pricing. He is said to favour a management approach that encourages cost-cutting by not giving mission designers a specific dollar amount to design to.

As a result, even though many scientists would prefer NASA simply to impose a single cost ceiling and stick to it, the agency is unlikely to do so. That leaves the research community — and NASA managers — with a dilemma: how to obey Goldin's 'cheaper, faster, better' dictum, when 'cheaper' and 'faster' are relatively easy to quantify, but 'better' is not.

Tony Reichhardt

Chile offers cash for ESO telescope site

Munich. The government of Chile last week agreed to pay up to \$12 million to the Latorre family, which claims to own the land on which the European Southern Observatory (ESO) is planning to build its Very Large Telescope (VLT), to settle a lawsuit that has been threatening the continued construction of the telescope.

Two independent assessors have been given a month to place a value on the 825-square-kilometre site, including the top of Mount Paranal, which is in the middle of a desert in northern Chile.

The family has in turn agreed to suspend a lawsuit over the land — and thus its demand for \$33 million in compensation — until the evaluation has been completed. The family is said to be prepared to accept \$12 million, and the government says it is prepared to pay up to this figure, depending on the outcome of the valuers' reports.

The agreement represents the first formal indication by the Chilean government that it is prepared to accept responsibility for settling the two-year-old dispute, which has led to injunctions being issued against ESO to stop building work on the VLT (see *Nature* 370, 494; 1994). It also takes some of the immediate pressure off ESO, which had been considering other sites for the telescope, despite the considerable investment it has already made in Chile. **Alison Abbott**

Japan 'will raise its LHC support'

Munich. Japan may pay up to half of the costs needed to bring forward the construction of the Large Hadron Collider (LHC) at CERN, the European Laboratory for Particle Physics, to CERN's initial completion date of 2005.

Kaoru Yosano, Japan's minister of education, science and culture, told a meeting of the CERN council in Geneva on 23 June that his government was prepared to add significantly to the ¥5 billion (SFr68 million) it has already committed towards the construction of the LHC, the laboratory's next particle accelerator (see *Nature* 375, 169; 1995).

Yosano, who was attending the council meeting as an official observer for the first time following an agreement reached in Tokyo earlier this year, said later at a press conference that Japan is prepared in

principle to contribute "three to four times the amount [it] has already given to support the LHC as it develops".

The statement has provided a boost to CERN's director general, Christopher Llewellyn Smith, who was able to obtain approval for the LHC from CERN's member states only by extending the original timetable for construction and experiments, and agreeing that these should take place in two stages to keep costs down.

A return to the original timetable for construction, under which top energy (14 TeV) would be achieved by 2005, would require a further SFr500 million (US\$575 million) in contributions from non-member countries. It now looks as though half of this could come from Japan.

Alison Abbott

Panel seeks cut and delay in international fusion project

Washington. An international collaboration aimed at proving that fusion power can work should be radically scaled down to reduce its construction cost from between \$10 and \$13 billion to less than \$4 billion, according to a panel appointed by President Bill Clinton to assess the US fusion programme.

But as the United States' partners in the International Thermonuclear Experimental Reactor (ITER) — Russia, Europe and Japan — reeled from the panel's conclusions, congressional sources warned that the United States was unlikely to support fusion research, even at the reduced level recommended by the panel.

The panel is a subgroup of the President's Committee of Advisors on Science and Technology, and is chaired by John Holdren of the University of California at Berkeley. It says in a draft report that there is no realistic chance of the US fusion budget growing from its current annual level of \$370 million, but argues that a stable budget of \$320 million could preserve "the most indispensable elements" of the programme.

The draft calls on the administration to "immediately open negotiations with the ITER partners" to scale down the planned international facility from one that could sustain fusion continuously to one which could manage it for 100 seconds at a time. It says, too, that construction should be delayed for three years.

The panel also suggests a domestic programme that would enable the Princeton Plasma Physics Laboratory (PPPL) to extend the life of its Tokamak Fusion Test

Reactor (TFTR) for three years. US officials would then reassess the need for its replacement, the Tokamak Physics Experiment.

Responding to the draft report, circulated last week in what may be a vain attempt to influence this year's budget process in Congress, Anne Davies, head of fusion at the Department of Energy, said she favoured its recommendations. But she added: "the first issue for the administration to decide is whether it can afford to put \$320 million a year into fusion for ten years".

The main issue in ITER, she said, was that the United States would have to "cap" its contribution. Scaling back the programme is not the only way to do this; alternatives include attracting more partners, or getting the country that eventually hosts the facility to pay a larger share, said Davies.

Robert Aymar, director of ITER, declined to comment on the Holdren report, saying that it is only a draft. But Davies claims that the international partners are "very upset" by the report.

Ron Davidson, director of PPPL, said he was "very encouraged" by the Holdren report, which, he said, "made a very strong argument that the fusion effort should be sustained at \$320 million". But congressional staff say the report had come too late to save the fusion programme.

The House Appropriations Committee has already agreed to slash this year's fusion budget to \$229 million (see *Nature* 375, 662; 1995). There are few signs that the relevant Senate subcommittee will try to restore much of that cut.

Colin Macilwain

NIH review to suggest streamlined handling of gene therapy bids

Washington. An external advisory panel has provisionally concluded that the Recombinant DNA Advisory Committee (RAC) of the US National Institutes of Health (NIH) need not review human gene therapy protocols that are highly similar to protocols already approved.

Initial screening of applications would be made within the Office of Recombinant DNA Activities (ORDA) on the basis of standardized information that would be required with each application. This data would include the rationale for the trial, any similarities to other trials already approved by the RAC, details of the vector and its target cells, and criteria for patient selection.

ORDA itself would, after a preliminary review, pass directly to the Food and Drug Administration applications similar to protocols already approved by the RAC. Other completed applications would be scrutinized by the full RAC.

At a meeting last week in Bethesda, the panel of 11 non-NIH scientists also reached a preliminary conclusion that the current balance of the RAC, at least 14 of whose 25 members must be scientists and at least six non-scientists, is correct, and that the number of scientists should not be increased.

The *ad hoc* review committee was created in March by Harold Varmus, the director of NIH, to review the role and structure of the RAC; it is due to make its final report by November. The panel, chaired by Inder Verma of the Salk Institute, in San Diego, is working in parallel to another group, chaired by Arno Motulsky of the University of Washington and Stuart Orkin of Harvard Medical School, which is looking at the entire NIH effort in gene therapy.

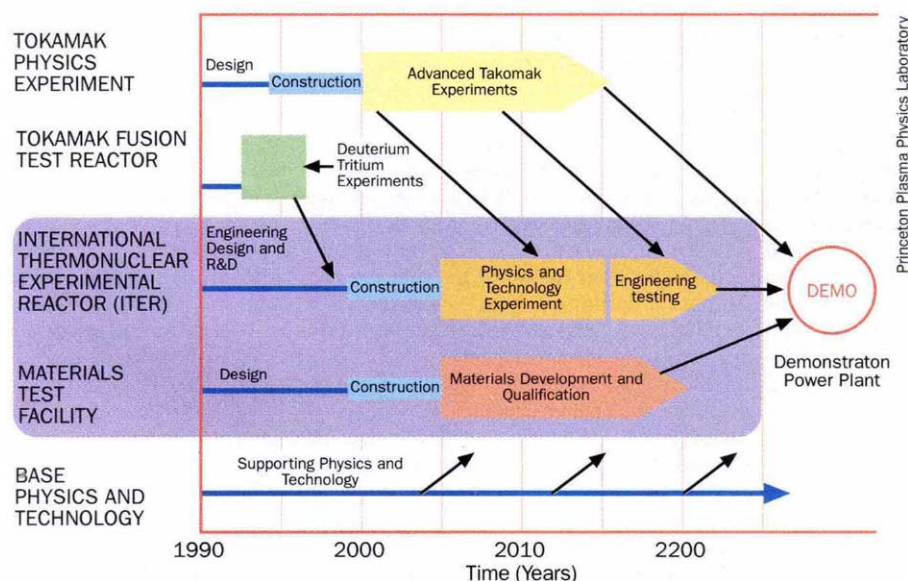
Verma points out that many researchers, such as gene therapy pioneer W. French Anderson, as well as representatives of the biotechnology industry, have argued that the RAC should only review proposals that break new ground.

But he says that many members of the review panel — as well as of RAC itself — believe that most proposals for human genetic therapy should continue to be discussed in the kind of public forum that the RAC provides. Indeed, according to Verma, the panel has yet to agree on the broadest question before it, namely how the role of the RAC should evolve as experiments involving the genetically altered cells in humans become increasingly routine.

Many panel members, he says, are keen that the RAC should continue to evaluate the scientific quality of proposals that come before it, as well as anticipating the difficult policy issues likely to arise in the relatively near future.

Robert Taylor

The US route to a working fusion power plant



The United States' original strategy (above) to make fusion feasible must be curtailed, the Holdren panel says — but its "most indispensable elements" can be retained.

UK to set up advisory panel on genetic data

London. Britain seems likely to become the first country in the world to set up a national advisory committee to provide government ministers with advice on how they should regulate the use of genetic information.

This follows an announcement last week by Virginia Bottomley, the Secretary of Health, that such a committee — which would be non-statutory — may be set up after consultation with clinical geneticists, scientists, manufacturers of diagnostic tests and other products, and the public.

Various European countries have already introduced legislation placing strict limits on the way in which genetic information can be

used. In several cases — as in US states such as California (see *Nature* 371, 468; 1994) — these have included a prohibition on the use of this information by insurance companies.

Bottomley's initiative appears to eschew this strategy by suggesting that an advisory committee "which would keep in touch with developments and act as a source of general advice to ministers" would be preferable to highly restrictive regulation.

Indeed, some observers say last week's announcement, whose tentative wording suggests that the idea is still in gestation in Whitehall, may be an attempt to forestall calls for even stricter measures in the report

of a House of Commons committee due to be published in the next few weeks.

The government's move was announced in a speech by Bottomley outlining what she considered to be the various successes of reforms to the National Health Service introduced over the past three years, and widely considered as her 'swan-song' before being moved to another department.

Acknowledging that developments in genetic testing will increasingly enable individuals to learn whether they are susceptible to certain diseases, Bottomley said it is clear that the government "has a public health and consumer protection role to ensure that such tests are supplied and used ethically".

She added that individuals need to be prepared for the results and, if they are abnormal, "properly advised on the implications" — a reference to widespread concern over the problems that could arise following genetic screening if it is not backed by procedures designed to supply such advice. Recognizing the privacy issues involved, she also said that such information "needs to be protected".

The government's plans, although still at a preliminary stage, have been welcomed by members of the Nuffield Council on Bioethics. This acts as Britain's national bioethics committee, and published a report on genetic screening in 1993 asking for such a committee to be set up. "Bottomley's suggestion is very much in line with what we recommended," David Shapiro, the executive secretary of the council, said last week.

The move has also been welcomed by those particularly concerned about the pressures that the availability of genetic information will inevitably place on insurance companies to take such information into account in setting life and health insurance premiums.

"Setting up this committee at this time would get very wide support from all quarters — including, I suspect, the insurance industry," says Peter Harper, chairman of the Royal College of Physicians' clinical genetics committee.

At the same time, however, there is likely to be intense debate over who should be represented on such a committee, and in what capacity. Alastair Kent, for example, the executive director of the Genetics Interest Group, a lobbying organization, says that those who sit on any new committee should be "mindful of the need to cover as wide a range of views as possible".

Specific proposals on how such a committee might operate are likely to come in the report on human genetics from the House of Commons Select Committee on Science and Technology, which, over the past year, has been studying a wide range of issues relating to the social implications of human genetics.

David Dickson

Curtain to fall on Beckenham lab

London. Glaxo Wellcome, the world's largest pharmaceutical company following the £9.4 billion (US\$15.0 billion) takeover of Wellcome by Glaxo in March, has announced plans to close the former Wellcome UK research site at Beckenham, outside London, over the next three years.

The new company will concentrate all its British research at its recently opened £700-million Medicines Research Centre at Stevenage, north of London (see *Nature* 374, 756; 1995). Development work will take place mainly at Ware in Hertfordshire and Greenford in Middlesex.

The decision to close the Beckenham site will affect about 1,550 jobs, and brings down the curtain on one of Britain's most significant industrial research sites. The site, where the company's Wellcome Research Laboratories were originally opened in 1923, boasts five Nobel prizewinners, five Queen's awards for technological achievement, and a major role in the development of important drugs such as the AIDS treatment zidovudine.

But the closure was not wholly unexpected in the wake of Glaxo's sharply contested takeover of Wellcome. Indeed, the industry has expected jobs to disappear as the combined company pursues the cost-saving that Glaxo admits was one of the main motives behind its purchase of Wellcome.

Furthermore, last week's announcement may be only the tip of the iceberg. Market analysts believe that between 10,000 and 15,000 jobs could eventually go in the process of combining the two companies.

Glaxo Wellcome says the Beckenham closure is part of a strategy to increase efficiency and eliminate duplication in an industry that is changing at an unprecedented rate. "It is part of the process of creating a world-leading pharmaceutical company capable of competing in markets globally," says a company spokeswoman.

The precise number of jobs to be lost remains unclear. Of the 1,300 research and development staff at Beckenham, a significant number are expected to be retained by the new research and development organization. The decision to locate the whole of the new company's UK research at Stevenage has been based on the desire to get scientists working together in a state-of-the-art environment.

Stevenage has space to accommodate about 1,500 scientists, and is already home to 1,000 Glaxo researchers. Although no accurate breakdown is available, 500 of the Beckenham employees are believed to be engaged in research work, and 800 in product development.

At the end of the process, it is therefore likely that Stevenage will be at full capacity. Glaxo Wellcome remains coy about the likely number of redundancies, as the selection process for the new research and development organization, aimed at retaining what a spokeswoman describes as "the most appropriate people from the two former companies", has not yet been completed.

Employees are expected to know their fate by the end of September. The company is reluctant to speculate about how many may be asked to leave, arguing that it is concerned about the likely reaction of those asked to relocate.

But trade union officials fear the worst. Indeed, Paul Talbot, national pharmaceuticals officer for the union MSF (Manufacturing, Science, Finance), is already warning of likely resistance to compulsory redundancies, and is urging the company to give former Wellcome researchers sufficient time to weigh up relocation versus voluntary redundancy.

There is also considerable concern over the impact of closure on Beckenham. One estimate is that over £100 million will be lost from the local economy each year after Wellcome's researchers leave. Mike Ward

Is metal disposal toxic to deep oceans?

E. G. Nisbet and C. M. R. Fowler

THE controversy over the proposal by the Shell Oil Company to sink the Brent Spar oil storage platform in the North Atlantic — and the company's decision to abandon this plan under pressure from the environmentalist group Greenpeace — centres on the question of the toxicity of metals in the tanks and ballast of the platform.

The metals involved are said to include "hundreds of kilograms" of cadmium, mercury, zinc, lead and nickel, as well as varying amounts of aluminium, copper, radioactive material and steel, 40 tonnes of oil and 100 tonnes of silt¹.

To put these quantities in context, estimates of metals released by natural systems in the Broken Spur hydrothermal vent field in the North Atlantic range from 500,000 to 5 million tonnes a year². In other words, a single vent field can emit annually orders of magnitude more of each heavy metal than is contained in the Brent Spar.

Ocean-floor sediment lying on new mid-ocean crust can be extremely rich in metals. For example, in the NESCA area in the northeast Pacific, a small area measuring 600 metres by 1,200 metres was found to contain 1–1.5 tonnes of uranium within its top metre of sediment, as well as about 4,000 tonnes of magnesium. Elements enriched in the sediments also included iron, manganese, aluminium, silver, barium, chromium, copper, lithium, nickel, lead, strontium, vanadium, zinc and rare-earths³.

Cadmium is another element characteristically enriched in sediments at mid-ocean ridges, and found for instance on the Gorda Ridge⁴ in the Pacific, and around the TAG hydrothermal mound in the North Atlantic. Locally, surface sediment is 20–40 per cent iron⁵.

A thriving biological community exists around many metal-rich deposits, and for some ecosystems around mid-ocean ridges a supply of heavy metals is essential. Bacterial mats on polymetal sulphide surfaces appear to use as their chemolithotrophic electron or energy sources either dissolved minerals or solid metal sulphides⁶.

The hydrothermal supply may ultimately introduce into the oceans some of the iron, manganese and sulphur that are essential to planktonic photosynthesis, as well as metals for important metalloproteins in marine life, such as nickel in urease, and zinc, copper, cobalt, molybdenum, mercury and cadmium in various planktonic proteins.

Finally, oils are produced by some sediment-covered systems, for example in the Guaymas Basin in the Gulf of California.

Here, in addition to metals such as iron, lead, zinc, copper, arsenic, cadmium, manganese and antimony⁷, a condensate contains C₁–C₂₀ hydrocarbons and polynuclear aromatic hydrocarbons such as phenanthrene, anthracene, fluoranthene, pyrene, benzantracene, chrysene, and benzofluoranthene⁸, made *in situ* by ridge processes.

The ocean floor is diverse, and we can distinguish between shallow seas, deep ocean floor and near-ridge settings. In shallow seas, the contents of the Brent Spar would indeed probably damage local marine life. In contrast, in the special environment around mid-ocean-ridge hydrothermal systems, the arrival of the metals of the Brent Spar would not be out of the ordinary, and indeed might even be beneficial as a mimic of vent activity.

The amounts of metals on the Brent Spar would be small here compared to local geological sources of elements, from magnesium to uranium, and local sediment would typically be metalliferous. As a result, the addition of extra dumped metals would probably act as nutrient to the local ecosystem.

In deeper seas, where the planned disposal of the Brent Spar was to have occurred, localized off-ridge venting occurs, and locally bottom conditions may occasionally be metal rich. As a result, the environmental damage from the disposal of the Brent Spar in this setting would probably be minimal.

The alternative to dumping on the deep ocean floor is to dispose of the heavy metals from the Brent Spar on land. But this will be problematic, as it will involve a difficult breaking-up process, followed by storage in a landfill, where aquifers may become contaminated. To land biota, elements such as cadmium can be very toxic.

We should be cautious of excessively anthropomorphic thinking; one man's poison is a bacterium's meat. A suitable disposal site could be in a carefully chosen location on the Mid-Atlantic Ridge, where the materials of Brent Spar would not necessarily be toxic to the local ecology.

Ironically, the recent G7 meeting of world leaders, at which the Brent Spar was discussed, took place within sight of one of the world's great marine research institutions, the Bedford Institute of Oceanography in Dartmouth, Nova Scotia. Had advice of scientists at the institute been asked for at the meeting, the Brent

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Life and depth: A black smoker in the hydrothermal vent field on the Juan de Fuca Ridge (northeast Pacific) at a depth of 2,200 metres. The emerging fluid is at over 350 °C, and 'smoke' is formed when contact with surrounding cold seawater causes metal sulphides to precipitate. Vent animals adapted to these conditions, such as white vestimentiferan tubeworms, grey limpets and several polychaete worm species, live in a steep temperature gradient within centimetres of this fluid. Tentacles at upper right belong to alvinellid polychaetes, which build tubes of metal sulphide particles and have high concentrations of elements such as cadmium, mercury and uranium in their secreted mucus⁹. The view illustrated is about three metres in width.

Spar affair might have been dealt with very differently and attention could have been focused on more important issues.

Arguably the most pressing environmental problem is overfishing in the North Atlantic, which urgently needs the wise political leadership that could have emerged from the Halifax meeting. By concentrating on and sensationalizing relatively small problems, we risk making poor judgements and neglecting more serious issues facing the environment. □

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1. Linklater, M. *The Times* 20 June 1995.
2. Nesbitt, R. W. & Murton, B. J. *BRIDGE News* **8**, 35–37 (1995).
3. German, C. R. *et al. Chem. Geol.* **119**, 175–190 (1995).
4. Koski, R. A. *et al. Can. Mineralogist* **26**, 655–673 (1988).
5. German, C. R. *et al. J. geophys. Res.* **98**, 9683–9692 (1993).
6. Wirsén, C. O. *et al. J. geophys. Res.* **98**, 9692–9703 (1993).
7. Peter, J. M. & Scott, S. D. *Can. Mineralogist* **26**, 567–588 (1988).
8. Gieskes, J. M. *et al. Can. Mineralogist* **26**, 589–602 (1988).
9. Juniper, S. K. *et al. Deep Sea Res.* **33**, 339–347 (1986).

UK and space science

SIR — The European Space Agency (ESA) faces difficult decisions, as indicated by your recent news report¹ and the correspondence from Bleeker and Woltjer². They, of course, are the architects of ESA's Horizon 2000 and 2000+ programmes, the troubled centrepieces of ESA's flagship scientific programme.

You have already reported that the United Kingdom has been unable to contribute planned instrumentation hardware to the gamma-ray satellite Integral^{3,4}. The reconfiguration of Integral's payload arrangements is not yet assured. Italy has offered to fill the gap left by the United Kingdom. But Italy is, as a consequence, in difficulties over its intended contribution to the UK-led optical Monitor instrument for the XMM mission.

Following advice from ESA, this instrument has been de-scoped and reconfigured (with a proposed small extra contribution from the United Kingdom which, happily, we are able to make). It is generally known in the space community that Germany has been negotiating a reduced contribution both to Integral and, like the United Kingdom, to Rosetta.

ESA's science directorate is responding with imaginative *ad hoc* arrangements that are producing real savings in the procurement of the instrumental payloads of XMM, Integral and Rosetta; studies are being carried out to determine how savings can be made on the larger costs associated with the spacecraft.

But the structural problem remains: European space scientists are progressively unable to make the 'in kind' contributions to the ESA space science payloads that have been planned. In some cases they have also been unable to afford the ESA space science subscription. The balance of the ESA programme has gone wrong, with national contributions under increasing strain because of the increase in subscriptions in the past decade and the tough economic climate in Europe.

Like Bleeker and Woltjer, we should all be concerned at the effects of this imbalance on our creative young scientists and engineers, who are supported by the national funds associated with the ESA subscription.

Britain is not alone in its difficulty. ESA has already shown its flexibility in responding to requests from Spain and Ireland for special measures to alleviate their space science subscriptions to ESA. Like the United Kingdom, Germany wants to reduce its subscription, and other countries have their own concerns.

At the same time, ESA member states have become increasingly aware that the agency cannot deliver everything they want. France is proposing to set up a small satellite science programme outside the

ESA framework. Germany and Italy, like the United Kingdom, are looking at national and bilateral space missions as an inexpensive complement to ESA's big missions. The Scandinavian countries are investing in their own national space programmes.

ESA states are also aware that the US National Aeronautics and Space Administration (NASA), which has gone through a very difficult time, is putting reforms in place with a 'smaller, cheaper, faster' policy for missions that is intended to make the agency more effective.

The ESA Council is recently reported to have commissioned consultants to study how the agency can make itself more efficient, particularly in its handling of industrial contracts and in its management practices⁵. No figure has yet been revealed to indicate how much the proposed reforms might reduce ESA's costs. But the conclusions and recommendations of the study are far-reaching.

Professor Mike Cruise¹ estimates that, even if ESA continues with its present industrial policy, the science programme can save 10–15 per cent on the STEP satellite mission, which he is proposing to ESA. On the basis of earlier studies and comparisons with national missions — and using the most recent studies for confirmation — the United Kingdom has set a target of 25 per cent for the savings that, altogether, it believes are possible, and which Bleeker and Woltjer assert are not.

They point with justifiable pride to the achievements of the ESA science programme. British scientists are equally proud to have played their part in this success story. To ensure their continued part in this success, and aware that we need our European colleagues equipped to work with us, the United Kingdom has put forward proposals for savings and changes in procedures in the ESA science programme. We believe our proposals will restore the balance between the national and international components of European space science, producing a leaner and more responsive space science programme with the same scientific output.

The future of ESA depends on accepting the challenge of competition in the real world. Is it really true, as Bleeker and Woltjer suggest, that these perturbations will have a "devastating effect" on ESA's space science programme? If so, the future is certainly bleak, as these perturbations are surely here already.

We need not just a blanket denial of the situation, but a positive response to the challenge of the conditions of the millennium years to ensure that European space science fulfils the future promised by Horizon 2000 and 2000+. Such a response is coming, not only from the space sci-

tists of Europe — including the United Kingdom — confident in the future of both ESA and space science, and working together to create that future, but also from those in the ESA family prepared to change the severe external constraints under which the ESA science programme has to operate.

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SIR — In "Britain gets a rough ride over call for space cuts"¹, I was extensively quoted. The article accurately reflects my views, but not the location of the institute at which I work. Given the political heat being generated over the issue, I am not sure to which institute you should apologize but, to set the account straight, I am based at the Rutherford Appleton Laboratory, not at the University of Sheffield.

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1. Dickson, D. *Nature* **375**, 441 (1995).
2. Woltjer, L. & Bleeker, J. *Nature* **375**, 624 (1995).
3. Dickson, D. *Nature* **373**, 459 (1995).
4. Pounds, K. *Nature* **374**, 491 (1995).
5. de Selding, P. B. *Space News* 31 May 1995.

Worthy journals

SIR — You defend (*Nature* **375**, 11; 1995) the proliferation of scientific journals on the grounds that on browsing through some randomly selected issues, you found articles of interest in all of them.

I cannot assess the status of the journals in the physical sciences at which you looked. But it is striking that the two biomedical journals included *The Lancet* and *The New England Journal of Medicine*, which are, unquestionably, top-ranking serials in the field. It would be surprising, therefore, if every issue did not contain something worth noting.

But this is not in itself the issue of concern about the continuing emergence of new titles. There is a finite limit to the number of specialist journals that can coexist before standards of reviewing and data reliability become noticeably reduced. One could argue that this is itself damaging to the long-term integrity of the scientific process. On a more pragmatic level, the more competition for limited subscription funds between journals, the less efficient the publication process becomes. Surely this at least partly underlies the startling rate at which subscription prices are outstripping the capacity of libraries to sustain their holdings.

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Science in an era of political change

With peace under way, and bright immigrants pouring into the country, Israel has a huge opportunity to maximize the potential of its strong science and technology base. Get it right, and its future will never have been brighter.

THE historic handshake between Yitzhak Rabin, Israel's prime minister, and Yasser Arafat, the leader of the Palestinian Liberation Organization (PLO), promises to bring peace to the Middle East. It is now being followed by another handshake, this time between peace and science, that promises to help bring prosperity also.

On the one hand, scientific coopera-

and foreign investment.

Economics, not politics, is what mainly affects research support in Israel, says Haim Harari, the president of the Weizmann Institute, as there is otherwise broad political consensus that research and education are important. He predicts that the outcome of the 1996 general election will have little direct effect on research provided that growth continues.

⌘ The current period of economic growth, he adds, means that "now is a very good period for science".

The biggest effect of the peace process on Israel's economy is likely to be the decision by the six countries of the Gulf Cooperation Council to end the so-called secondary and tertiary "Arab boycotts", which prohibited trade with companies doing business either with Israel

or with other companies trading in Israel.

The decision, announced last year at the first Middle East/North African Summit in Casablanca, in which Israel also participated, will lead to a massive increase in foreign investment, predicts Shuki Gleitman, chief scientist at the ministry of industry and trade, in particular from countries such as Japan which depend heavily on Arab oil.

The lifting of restrictions by Arab countries on direct trade with Israel, also announced at the summit, will also lead to greater regional cooperation on gas, water and electricity infrastructures, but is itself unlikely to have much effect on Israel's economy.

More than 60 countries that previously avoided doing business with Israel have now opened diplomatic and economic relations. Many of these countries are also new emerging economies in Asia and the Pacific Rim, and Israel, which has until now been excluded from around two-thirds of the world's markets, has an opportunity to go on a selling spree.

Moreover, economic analysts reckon that what Israel will mostly be selling will be high-technology products, which have increased their share of Israel's exports from less than 40 per cent in 1989 to more than half last year.

But if Israel is to take advantage of these opportunities, it will need to maintain its strong science base, radically improve its

systems for technology transfer and address the notorious weakness of its companies in marketing and selling technologies in the international market. □

Science and peace

SCIENTIFIC cooperation between researchers in Israel and their colleagues in neighbouring Arab states is making an important contribution to political efforts to bring peace and stability to the region, by helping to bring down the barricades that have separated their countries for almost half a century.

Seminars on "science and peace" have become almost a fashion. In April, for example, the "First Israeli-Arab Scientific Meeting" was organized in Paris, by the prosaically titled "French Association on Behalf of Cultural and Scientific Exchanges between Middle-Eastern Peoples". In May, a "Conference on the Peace Process and the Environment" was also organized at Tel Aviv University.

"Science is less political than other issues, it's a bridge for peace", says Leah Boehm, chief scientist at Israel's science ministry, on her way to a meeting aimed at launching a programme to enable Jordanian postgraduates to take PhDs in Israel.

Avi Golan, a researcher at the Jacob Blaustein Institute for Desert Research at Sede Boker, has also recently begun cooperating with Egyptian researchers on the biochemistry of desert plants. "It's fantastic," he says, "I fought against

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tion between Israel and its Arab neighbours is reinforcing peace by contributing to dialogue, and also by helping to replace national competition with regional cooperation aimed at better management of scarce resources, protecting the environment and fighting disease.

The peace process, in turn, promises to benefit the entire research enterprise both in Israel and throughout the Middle East. Immediate returns will come from the extra funds that will flow towards joint Arab-Israeli projects from foreign countries and organizations with an interest in sustaining peace.

But the major benefits of peace to science and technology will probably be more indirect, and will result from the expected increase in economic growth

Science in Israel

This survey has been written by *Nature's* European Correspondent, Declan Butler, who wishes to thank Paul Ogden and his staff at the Weizmann Institute, Esther Koslovsky at Tel Aviv University, Jerry Barach at Hebrew University, and Uri Stein at the science ministry, for their help in arranging interviews, often at very short notice. Thanks also to Sonia Chanaz for editorial assistance, and Baruch Raz and Dori Goren at the Embassy of Israel in Paris, for help in making arrangements. Apologies to those whose work, for reasons of space, has not been described in detail.



Bethlehem University.

Egypt in the 1967 war, now we're working together."

But when Israelis speak of cooperation, some Arab scientists fear that this will be fuelled by Israeli brains, outside money and Arab 'technicians'. The basis of their fears is a sensitive issue, but can be simply stated. Israel has spent much more on science than its Arab neighbours, and is more advanced in almost all areas of science.

What is true for Israel's established Arab neighbours is even more true for the West Bank. "We are at a big disadvantage, we don't have the universities, infrastructure, or money that the Israelis have," admits one West Bank scientist.

He argues that cooperation "is a must", not only to help peace but to help Palestinian universities develop, but says that "if the Israelis want cooperation, they should open their universities to us. We have the academic talent, cooperation has to be symmetrical".

Such arguments hold little sway with some Israeli universities, however. "The Palestinians want help to develop, but we don't have the resources for this," says Hanoch Gutfreund, president of Hebrew University in Jerusalem. "We don't even have that sort of collaboration with Tel Aviv University, we're not here to help universities compete with each other."

Cooperation is a "breakthrough", says Gutfreund, "that is important for the region". But he argues that there is no point yet in collaborating in areas of high technology. Instead, he favours forms of cooperation where Israelis and Arabs can be "on an equal basis", and where projects are of regional interest and also likely to attract international funding.

Indeed, apart from the initiatives of individual researchers, the promise of international funding seems the biggest carrot for cooperation. The United Nations Educational, Scientific and Cultural Organization (UNESCO), for example, has agreed to fund a plant biotechnology centre at the Bethlehem University that will also involve Israeli researchers.

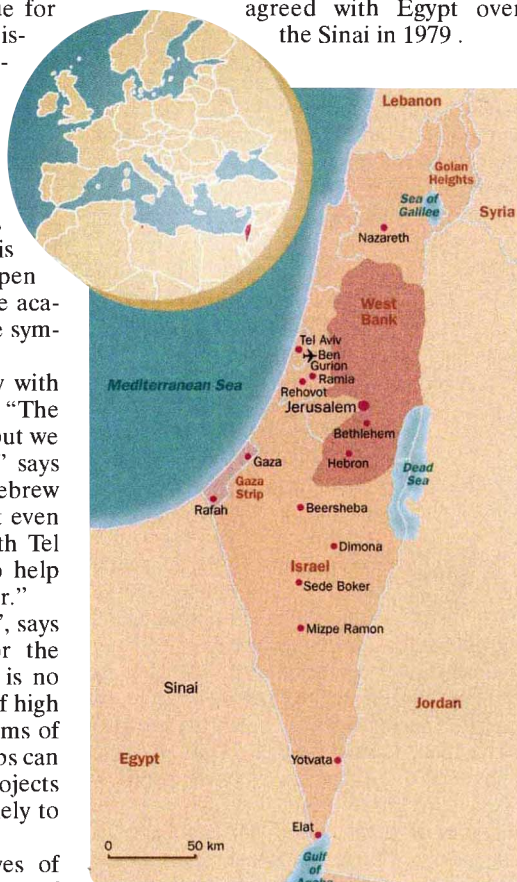
Boehm, for her part, is chasing funds from the United States and Germany to set up a programme with Jordan aimed at studying environmental protection, irrigation, water remediation and insect problems in the Jordan Rift Valley. With two sons serving as paratroopers, one of whom is stationed in Lebanon, she admits to having more than a professional interest in peace. □

The politics of peace

PEACE talks between Israel and the neighbouring Arab states began with the Madrid conference in 1991. Since then Israel has signed a framework for peace with the PLO, made peace with Jordan

after 46 years of hostility and opened discreet diplomatic relations with other Arab countries.

The key to stability in the region will depend on Syria and Lebanon making peace with Israel. Last month, Israel accepted that it should leave the Golan Heights, opening the way to negotiations with Syria that could lead to Israel's withdrawal from the area in a similar "peace-for-land" settlement to that agreed with Egypt over the Sinai in 1979.



Jerusalem remains the joker in the peace process pack. Radical Palestinians will not settle for less than Jerusalem as their capital, while Israel is unlikely ever to compromise on giving back parts of Jerusalem.

But the future of the peace process will depend mainly on the outcome of the 1996 elections, when Israelis will elect 120 representatives to the parliament, or Knesset, under a system of proportional representation.

No party has ever obtained a majority in the Knesset, and horsetrading to form coalition governments is the rule. Coalition governments led by the Labor party, a social-democratic group which originated from the pre-state Jewish leadership, ruled Israel from 1948 to 1977, when the right wing Likud (consolidation) party came to power. Labor regained power in 1992.

Likud is a coalition including Herut (Freedom) and the Liberal party, which historically has owed much of its success to its defence of disadvantaged

Sephardic Jews from the Middle East and Africa. But the party has turned towards the ideology of a 'Greater Israel' inherited from Herut, taking up the cause of the occupied territories and siding with the extreme right against Rabin's peace deal with the PLO.

Indeed, what distinguishes the two main parties is that Labor favours territorial compromise, while Likud does not. Otherwise, left and right have few economic connotations in Israel; both Labor and Likud support the free market, privatization and deregulation.

Recent polls showed the current prime minister, Yitzhak Rabin, of the Labor party lagging behind his Likud rival, Binyamin Netanyahu. This month, however, Likud was weakened after David Levy, Netanyahu's main rival in Likud, left to set up his own party. With Likud divided, a Labor victory in 1996 will mainly depend on Rabin convincing Israelis that the peace process is good for them. □

Occupational hazards

BETHLEHEM lies in the occupied territories just 20 minutes' drive south of Jerusalem. But the distance seems much larger for the Palestinians queueing in the sun either to obtain permits to enter Jerusalem or to cross the frontier checkpoint itself.

"Freedom of movement" remains the biggest problem for researchers in the West Bank, according to Moien Kanaan, the chairman of the department of life sciences at Bethlehem University. Kanaan himself has a Jerusalem identity card that allows him to cross the frontier as he pleases, except during the frequent periods when the Israeli Army closes it. Earlier this year, the border was sealed for several months, following attacks on Israel by Palestinian groups.

But many of Kanaan's colleagues face much greater difficulties because they need to obtain travel permits even for a short visit to the nearby Hebrew University in Jerusalem. "The policy for authorizing such permits remains 'completely arbitrary'", says Kanaan.

Depending on the mood of the official behind the window", he claims, "you will get a permit for a month, a week or just two days, and it can be cancelled any time the Israelis want." As a result of such difficulties, most researchers ask the university to apply for permits on their behalf, as it "carries more weight".

Kanaan admits that the peace talks are making it easier to get permits, and that border closures have become less frequent. But he complains that the time, effort and humiliation involved show that the Israelis still have the "mentality of an occupier". "Being a Palestinian is still a problem", he says. "The Israelis have yet to understand the dignity and human factor."



Moien N. Kanaan (above). "Freedom of movement" remains the biggest problem for West Bank scientists. Students at Bethlehem University (left) celebrating the victory of the pro-Arafat, pro-peace talks party in student elections last month.

Kanaan does not consider himself a political radical. His education, he says, allows him to entertain the idea of peace even though the benefits are not yet "felt in the streets". But he is concerned that many Israeli actions, such as last month's attempt to annex Arab land close to Jerusalem, make it more difficult for academic liberals like himself to defend their support for the peace talks.

The political situation also continues to inhibit Palestinian researchers from cooperating with their colleagues in Israel. "We want to cooperate, but why should we go and normalize our relationships when the Israelis here make our life miserable, when we can't cross the border to meet in Hebrew University which is just down the road?", asks one Palestinian scientist.

Such reasoning is also followed by the Palestinian Higher Council for Education, a body elected by the four West Bank universities. It recommends that normal relations with Israel should not be pushed too fast, given that political negotiations between Israel and the West Bank are far from complete. "It's too soon to go and hug everybody."

Despite the difficulties, Kanaan says he is "glad" to have returned to the West Bank three years ago, after having worked in laboratories in the United States for almost a decade. "I enjoyed the liberal way of life in the States, but I prefer to combine this with the community values we have here. I and my family are oriental in our hearts."

Returning has also meant making do on a monthly salary of US\$1,200, high by West Bank standards but difficult to live on nonetheless. Similarly, money and equipment for research are badly lacking at the university — a liberal school that focuses on teaching — especially in expensive disciplines such as molecular biology.

Kanaan himself researches the genet-

ics of local diseases, pointing out that the large extended families common in the West Bank are ideal for pedigree studies. He is also genotyping methicillin-resistant strains of *Staphylococcus aureus*, an

Refusenik researchers return

AN Israeli particle physicist was perplexed one day when the office cleaner suddenly asked to have a look at a calculation that the academic seemed unable to solve. The physicist was astonished a few minutes later when the cleaner, grinning like someone who has just finished someone else's crossword, handed him back the solution.

Whether this anecdote, variants of which are told from Dan to Beersheva, is true, is difficult to say. But it captures the situation of some of the 17,000 immigrant scientists and engineers who have arrived in Israel since 1989 but been unable to find jobs in their original professions. Indeed, at least one engineer does work as a cleaner at the science ministry itself.

Following the decision by the Soviet Union in 1989 to let its Jewish citizens emigrate, some 550,000 'refuseniks' have arrived in Israel, swelling its population by 10 per cent. Providing housing and jobs for such numbers — equivalent to the United States absorbing half the population of the United Kingdom over a period of four years — has demanded a huge national effort.

Such massive immigration, or *Aliya*, is nothing new to Israel. The state was created in 1948 by gathering in around 650,000 Jews from more than 100 countries of the diaspora. Over the next three years, 700,000 new immigrants arrived, and a similar number again during the 1950s. The "right to return" of any Jew is still taken for granted.

What distinguishes the latest wave of immigrants from those earlier ones is precisely that many are better educated or

epidemic strain that is a big problem in local hospitals.

"My eyes are hurting from writing grant applications", says Kanaan, who receives only a small research stipend from the university. The trick, he says, "is to examine the priorities of international funding agencies to find ones that match those of the West Bank."

The life sciences department has also recently obtained funding from UNESCO to build a plant biotechnology centre that will use tissue culture techniques to produce virus-free vines, for example, and also carry out research on drought-resistance genes. "The peace process is providing more opportunities for us to get money for research and travel abroad," says one researcher.

But although many Palestinian researchers welcome the increase in opportunities for foreign travel, many would nonetheless prefer improvements in travel restrictions to Israel. "It makes no sense for me to go to Europe every summer, when I could learn the same techniques down the road, and come home at night", points out one scientist. □

trained than the native population. Before 1989, Israel had the world's highest proportion of scientists or engineers — 130 per 10,000, compared with 77 in the United States, and 73 in Japan — and Soviet immigration has increased this number by more than half.

"It is the most important event since Israel's creation", say Zvi Yanai, the director general of the science ministry. "It's the biggest influx of scientists since the flood of scientists from Europe to the United States after the Second World War."

Indeed, the arrival of highly qualified scientists has boosted disciplines throughout universities and research institutes. The mathematicians are "outstanding-plus", says Haim Harari, the president of the Weizmann Institute, where immigrants at one point accounted for more than three-quarters of PhDs in mathematics. Similarly, Russian immigrants now account for a quarter of faculty in the mathematics institute at Tel Aviv University.

Immigrants have also been manna from heaven for the country's electronics and computer industries, which badly lack qualified engineers and scientists. "The immigrants really saved our companies," says one official, "by providing a timely stop-gap"; and also "cheap labour", say cynics.

But while the best scientists, or those whose skills match industrial needs, have easily found jobs, around 2,000 immigrants, many with good qualifications, have been unable to find jobs in their own areas, if at all. This is particularly true in the cash-strapped universities, which cannot afford

to support many new staff, and in hospitals. With 9,000 physicians, for example, Israel had more than it needed before immigration began. Similarly while electro-optical engineers are almost guaranteed a job, a railway engineer has little hope in a country where railways are not a priority.

The numbers of jobless immigrant scientists are also likely to rise in the coming months, as government support schemes, on which they have previously relied, begin to expire. "Getting money is a constant battle", says Harari.

The government has provided all immigrants with a package of cash and allowances, but then left them to sink or swim. Similarly, while the ministries of science and immigrant absorption have provided around 6,000 immigrant scientists with a small salary to enable them to "find their feet" within existing research group, this lasts only two to three years.

Eliezer Gileadi, professor of chemistry at Tel Aviv University, has led a campaign to persuade the government to support a new scheme to provide extra money for immigrant scientists. Otherwise, he argues, "all hell would break loose".

The Gileadi Plan, as it is officially known, would provide immigrant scientists with half the minimum monthly salary, about US\$1,200, and leave them to seek the remainder from other sources. In April, the government agreed to fund 300 such posts for three years, and Gileadi hopes it will agree to a further 200 and also make posts renewable until researchers reach retirement age.

"As the inventor of this programme, I have the moral right to say it's a bad programme, a compromise", says Gileadi. "We are privileged to have a brain drain towards Israel for once, but unfortunately can't maximize this advantage."

One area where the brain drain is being maximized is in intelligence. Immigrants have provided Israel with Russia's last secrets in space and nuclear technology, according to observers, and have supplied the information on materials needed by Israel to complete its Merkava tank.

Despite the many problems it would be a mistake to declare absorption a failure. "It was an amazing feat given the challenge", says Harari, who points out that many scientists in precarious positions will gradually climb the ladder to better jobs. □

Incubating immigrants

ISRAEL'S unique Kibbutz system of communal living has accumulated enormous debts, and is increasingly under fire for becoming a conservative lobby whose main aim is self-perpetuation at the expense of the taxpayer. This has done little, however, to dampen Israeli enthusiasm for innovative and large-scale social engineering.

Faced with the flood of immigrant scientists, the government has embarked on a

nationwide scheme to turn their expertise into research-based, export-orientated companies, in no less than two years, by setting up a series of "technology incubators" (TIs) across the country.

The incubators themselves are nonprofit umbrella structures, set up in association with either academic institutions, local councils or private companies, each of which houses several start-up companies. Immigrant scientists account for four-fifths of company staff, and for half of the business ideas. So far, 28 incubators have been established, housing a total of 240 new companies.

The idea of the incubator is to provide immigrants with a safety-net while they get to know the ropes of a market economy. The TIs themselves provide immigrants with training in negotiating skills, project management and free legal aid, and help them to find outside partners and investors. All the incubator's running expenses are covered by the ministry of industry and trade (MIT) up to a ceiling of 325,000 shekels a year.

To enter an incubator, immigrants must be "entrepreneurial", say Shuki Gleitman, chief scientist at MIT. The incubator then carries out a patent search on the immigrant's idea, and prepares a preliminary business plan for his company.

MIT also pays 85 per cent of each project's annual costs, including 100 per cent of salary costs and 75 per cent of other

expenses, up to a ceiling of 300,000 shekels, but entrepreneurs must find the remaining 15 per cent themselves. The scheme will cost MIT US\$33 million this year.

Such explicit government interventionism would bring tears to the eyes of even the most reasonable of free-marketeers. But a handkerchief is waiting. After only two years, all subsidies to the startup companies are withdrawn, and they must fend for themselves in the marketplace. Moreover, if they succeed, they must pay back MIT's original investment.

So far, 140 companies have reached this point, and 70 have survived, according to Gleitman. He boasts that Nanometre, a company created at the Technion incubator which has developed a motor of nanometric dimensions, is valued at US\$10 million. But he is also first to admit that "we don't have 70 Microsofts. This is not an employment solution; it's absorbing people and their technology."

But if many Israeli scientists are sceptical whether the incubators will make a major impact on the economy, the odd success story aside, most nonetheless applaud the scheme as an "imaginative and excellent solution to exploiting the potential of immigrant scientists". Indeed, one cynically suggests that a similar scheme should be used to "get the best scientists out of the universities and into industry". □

"At home in Zion"

To create the state of Israel, Zionists resurrected and revised a language that had been dead for almost 2,000 years. Learning Hebrew, at specially arranged crash courses, or *Ulpan*, is also one of the first tasks facing immigrants such as Isak Beilis, who arrived in Israel in 1991 after having been a senior research scientist at the Academy of Science's Institute for High Temperature in Moscow.

Beilis now works with a group of six other immigrants in the Faculty of Engineering at Tel Aviv University. The Electrical Discharge and Plasma Laboratory, which is led by R. Boxman, is developing vacuum arc techniques for rapid deposition of high-conductivity transparent coating, for uses such as flat-panel displays, solar cells and museum burglar alarms. It has also developed a process that speeds up the case-hardening of steel from one day to one-tenth of a second.

Their research on "arcs and sparks" is directly connected with the Hebrew word for electricity, points out Boxman proudly. Because electricity had not been discovered in biblical times, language revisionists had to invent a new term, and they chose "amber" or "Hashamal" in Hebrew, after the amber arcs in the clouds seen by Ezekiel during a vision.

Beilis, who is renowned for his research on electrode phenomena in vacuum arcs, and plasma physics, spent his first six months in Israel working in the laboratory without pay,



Isak Beilis, concerned for the future.

and making ends meet on a small stipend from the Jewish Agency. He has subsequently received around US\$1,000 per month, as part of a three-year Wolfson grant, one-third of the average salary of his colleagues.

Most of these grants expire soon, however, and Beilis admits that he is "concerned for the future". But he says he is nonetheless "happy to have had the opportunity to come and work in my field, that's the most important thing for me".

His wife, an electrical engineer, has been unable to find a job in her field, and now cares for elderly people. But the future for Beilis's teenage son seems bright. He has already taken third place in Israel's Mathematics Olympics, and been invited to join the Israeli team competing in the International Mathematics Olympics. □

Research organization: a coat of many colours

"ISRAEL is lucky not to have a centralized research system", says Haim Harari, president of the Weizmann Institute. "It's extremely dangerous in a small country to have central control." Harari does not risk losing sleep. The official diagram explaining who sets science policy and allocates funds in Israel looks as if its designer was experimenting with new flowchart software.

Eleven ministries have their own office of chief scientist, for example, most with a budget of around 3 million shekels, and each pursues its own policies. This decentralization results from the recommendations in 1968 of a commission chaired by Ephraim Katchalski-Katzir, a professor at the Weizmann Institute, and later the fourth president of Israel.

The defence ministry also carries out military research through its wholly owned armament development authority, while the prime minister's office controls the Israel Atomic Energy Commission. The National Council for Research and Development, which Katchalski-Katzir recommended should be responsible for setting national science policy, has ended up as an advisory body to the ministry of science and arts (MOSA).

The gravity needed to prevent the entire structure flying apart is supplied by the mass of ministry of industry and trade (MIT) and MOSA. MIT, in particular, has a massive budget, US\$450 million this year, for subsidizing industrial research, including 28 "technology incubators" aimed at absorbing immigrant scientists (see page 720).

MOSA lacks the financial muscle of MIT, but compensates by trying to coordinate almost anything with the word 'science' in it. The science minister, for example, chairs the ministerial committee for science and technology, and the chief scientists' forum. MOSA has also recently launched a national strategy for generic research (see below), and coordinates Israel's participation in international research organizations. The Israeli Space Agency (page 724) is also affiliated with MOSA.

The main source of funding for basic research is the Council for Higher Education (CHE), which has a total annual budget of around US\$1 billion. Most of this goes on salaries and overheads, however. Only about 15 per cent of a university's basic research budget comes from its own sources, including government allocations, and most researchers need to look elsewhere to obtain money for their research.

They are not spoilt for choice. Indeed, the proliferation of funding sources is such that each university has a 'research

authority' that brings funding opportunities to the attention of researchers, and reminds them of deadlines for proposals.

Hebrew University's research authority, for example, brings in about US\$41 million of external research funds annually. About three-quarters of its research is basic, and half of the remaining applied research is supported by company grants.

An effort to establish an analogue of the US National Science Foundation is also meeting with some success. Government funding for the Israel Academy of Sciences and Humanities, which administers Israel's National Science Foundation grants, should rise from US\$15 million last year to US\$20 million next year. Add the academy's US\$2 million income from private sources, and it becomes the biggest Israeli programme of its kind. It has already become the biggest source of funding at Tel Aviv University.

But most of the money available still comes from foundations established with foreign countries. The US Binational Science Foundation (BSF) was set up in 1972 with endowments of US\$30 million each from Israel and the United States. A US\$40 million increase in 1984 raised BSF's capital to US\$100 million, and this now yields around US\$7.5 million annually. To qualify for a grant one must have a bone fide collaborator in the United States.

A similar German-Israeli Foundation (GIF) was established in 1988, with DM150 million contributed by both governments. A United Kingdom-Israeli fund set up last year will also provide

£600,000 over three-years, while MOSA is also discussing the setting up a fund with China.

Earlier this year, US President Bill Clinton, and Yitzhak Rabin, Israel's prime minister, also created a US-Israel Science and Technology Commission to promote development of technologies and high-technology industries, and the conversion of military technologies to civil applications. Each government contributed US\$15 million over three years, and companies are expected to match this sum.

Besides these foundations, bilateral agreements with organizations such as the US National Institutes of Health provide additional funds. Germany's Max Planck

Society and the Weizmann Institute have also established the Minerva Foundation to administer a joint programme of research. Israel is also to join the European Union's Framework research programmes (see page 723).

Harari supports such diversified funding. "It's good to have to scramble for money", he adds, pointing out that the Weizmann Institute previously obtained only US\$4 million from industry, but now gets US\$13 million. "We wouldn't have achieved this if we had been fully funded by government."

But he nonetheless warns against Israel becoming too "dependent" on foreign money, and allowing this to influence research priorities. Hanoch Gutfreund, president of Hebrew University, argues, however, that the government should itself commit sufficient funds to meet basic research needs. □



Shulamit Aloni: science minister since 1993.

MOSA seeks promised land

ISRAEL'S ministry of science and arts (MOSA) is embarking on a scheme, similar to that endorsed in the United Kingdom last month by John Major, the prime minister (see *Nature* 375, 265; 1995), to focus its funding on "generic" priorities, in a bid to improve links between basic and industrial research.

Under the new scheme, MOSA will allocate US\$22 million this year to five generic priorities: computing, micro-electronics, electro-optics — where Israel already controls 90 per cent of the world market in some areas — as well as biotechnology, and material sciences.

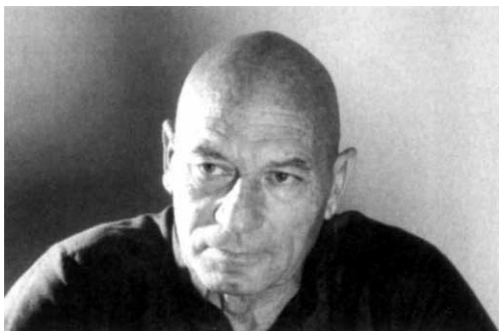
This sum will rise to US\$30 million next year, and US\$40 million the year after, and the number of priorities will be increased to include, for example, applied mathematics, environmental studies, water resources, pollution, social studies, or life sciences.

The move represents a bid by MOSA to use its funds more effectively. Although substantial, they account for only around 10 per cent of the country's total spending on research. MOSA previously took the politically expedient route of allocating money to research institutes to spend as they pleased, says Zvi Yanai,

director general at the ministry.

"We spread our resources too thinly, and when you spread thin you get zero", he says. MOSA will now award fewer grants, but will increase the amount of each individual grant from around US\$50,000 to US\$500,000, according to Leah Boehm, chief scientist at the ministry. "We are forcing scientists to think big, and to collaborate; we want to see a critical mass."

MOSA's overall aim is to support research that is more narrowly focused



Zvi Yanai, self-made man, and director general of science ministry.

than basic research, but either too new, generic, high-risk or long-term to obtain support from the ministry of industry and trade (MIT), which subsidizes industrial research.

Basic research in Israel is mainly supported by the Council for Higher Education (CHE), a 25-member commission chaired by the minister of education, whose powerful six-man planning and grants committee proposes and allocates the national budget for higher education and research. CHE has a total annual

budget of around US\$1 billion

Industrial research is supported by the chief scientist at the ministry of industry and trade (MIT), who has a budget this year of US\$450 million. MIT spending carries more weight than that of CHE, because most goes directly to research, whereas around 80 per cent of CHE's budget goes on salaries and overheads.

Moderate investment by MOSA could have "extraordinary leveraging power", by inserting an intermediate layer of generic research between the areas of basic and industrial research already supported by either of these two funding giants, claims Yanai. But such strategic research should not be at the expense of basic research, he adds.

Yanai points out that similar generic investment in defence — prompted by the French arms embargo imposed after the Six-Day War in 1967 — created Israel's electronics industry, which now accounts for more than half of Israel's exports. Given that the defence budget for strategic research and development has fallen by half over the past decade, Yanai argues that now is the time to develop a new strategy aimed at developing and exploiting new technologies.

This task cannot be left to industry, argues Itzhak Parnas, the head of the High Committee for Strategic Planning, which was set up last year to administer the new programme. Because MIT is allowed to fund only projects that private companies themselves invest in, industrial research has become too orientated towards existing industries, he argues. "If MIT had had this restriction 20 years



Leah Boehm: as chief scientist at the science ministry, she has a satellite view of the country's research.

ago, we would have invested in textiles not electronics."

Indeed, priorities are decided by Parnas's committee — which is made up of 13 industrialists, scientists and government officials — on the basis of Israel's strengths in these areas, their market potential, and whether technologies could be economically developed either by using existing industries or by creating new ones.

As evidence of the need for such a strategy, Parnas points out that while Israel has a strong science base in molecular biology, it lacks a "single excellent centre" for either protein microsequencing or the production of transgenic animals — including knock-out mice — and plants. "We will now build such centres", he asserts, "and fund them at the same level as in the US or Japan."

MOSA's initiative has been welcomed by Haim Harari, the president of the Weizmann Institute, who says that identifying priorities and funding them fully is the ministry's job. "Whether or not they choose the best priorities doesn't matter so much", he says, "but spreading money too thin is only a waste of money. If in this way they get extra money for research from the government, that's fine."

Indeed, while one researcher is critical of the scheme, which he says "puts all the ministry's eggs in one basket", many seem either enthusiastic or indifferent. One reason is that few rely completely, if at all, on MOSA for research funding, but more on international foundations.

One leading industrialist argues, however, that the scheme only confirms that the Israeli government's industrial policy still relies too much on "technology push", and not enough on market demand. The search for commercially successful technologies should be left to profit-seeking investors, he argues, not to government officials. □

Change at the top

THE decision by the ministry of science and arts (MOSA) to concentrate its funding on a few "strategic priorities" (see above) is due to a change of management at the ministry, and in particular, the arrival over the past two years of an energetic 'gang of four', who share firm convictions about the role it should play in shaping the country's research.

The minister herself, Shulamit Aloni, is the founder and leader of Meretz, a progressive-liberal party that is part of the ruling Labor coalition government. She is a founder of the Israel Consumer Rights Council, and an author on civic issues raised by religion, education and women's rights, and arts were included in the ministerial portfolio at her insistence, according to observers.

But the driving force behind the ministry's new policies is its director general, Zvi Yanai, a self-made man with little formal education, who established IBM

Israel's corporate communications department in 1970, and headed it for the next two decades.

The chief scientist, Leah Boehm, was appointed a few months ago. Despite being responsible for international foundations, policies on immigration and scientific cooperation with neighbouring Arab states, she still finds time — almost — to spend one day a week in her electro-optical laboratory at the Israel Atomic Energy Commission's centre at Soreq, near Rehovot.

Itzhak Parnas heads the ministry's high committee for strategic research, which was created last year to administer the new strategic programme. Parnas, who still works as a neurobiologist at Hebrew University in Jerusalem, also chairs the National Council for Research and Development, a forerunner of MOSA, which is made up of scientists, engineers and industrialists, and now acts as an advisory body to the ministry. □

Weizmann thinks big

"WEIZMANN is people-driven. If we have a top-notch person in an important field we will invest enough to be the best, if we don't, we won't invest, no matter how important the area is." This is how Haim Harari, president of the Weizmann Institute in Rehovot, Israel, describes the institute's research philosophy.

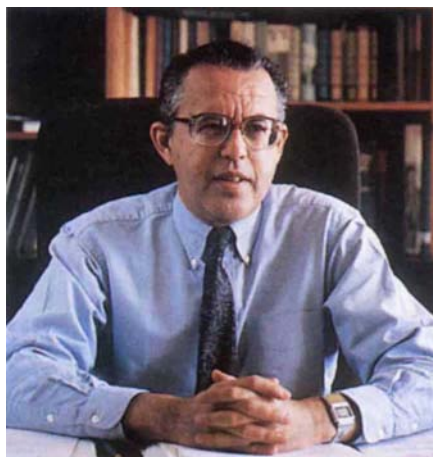
Harari, who is a leading particle physicist — he coined the terms 'top' and 'bottom' quarks in 1975, in a paper proposing the theoretical need for their existence — explains that part of the thinking behind this philosophy is the desire to counter the "main disease of Israeli science", namely the tendency to spread resources thinly, instead of concentrating them in a smaller number of larger projects.

Evidence of the Weizmann's investment strategy dot the 1.2 square-kilometre campus of lawns and subtropical gardens, kept green by a computer-controlled irrigation system — a US\$10-million heavy-ion accelerator built in the 1970s; a US\$15 million solar energy tower erected eight years ago and a US\$16-million nanotechnology centre constructed in 1990. A big brain-research centre is also being built. "No university in Israel has projects of similar scale", boasts Harari.

"In some areas, unless you invest enough, you needn't bother", says Harari. To build the nanotechnology centre, for example, the institute had the option of investing either US\$4–\$5 million or US\$16 million. The first choice would have been the easiest, but would "at best have duplicated work being done elsewhere".

The second option was a "risk and a gamble", says Harari, in particular because the US\$16-million funds would support "one brilliant person". "You have to make hard choices," he says, "and sometimes when you gamble you lose." Taking such choices is also the "best way to make enemies internally," he adds.

Indeed, the institute is unashamedly



Haim Harari, president of the Weizmann Institute.

elitist. "You don't become a good scientist by democracy, you are either at the front or get out", says Michael Sela, a renowned immunologist who was president of the Weizmann from 1975 to 1985. "There is nothing worse than being a mediocre scientist." Similarly, Harari claims that the institute abandoned research in high-temperature conductivity because "we didn't have a star".

But if the Weizmann has a superiority complex it is understandable. It produces some of the brightest stars in disciplines ranging from immunology to artificial intelligence. It is also continuing a tradition of vision and change inherited from its founder and first president, Chaim Weizmann, who later became Israel's first president.

Weizmann, a Russian-born chemist, believed that Israel's self-determination and self-sufficiency depended on linking science to Zionism, given that Israel badly lacked natural resources on which to build an economy. In 1933 — 15 years before the state of Israel was created — he founded in Rehovot the Daniel Sieff Research Institute, named after the son of Israel Sieff, a member of the family that owns the British Marks and Spencer chain of department stores.

At the time, Rehovot was only a small agricultural community in the British Mandate of Palestine. The Jewish population of Palestine numbered fewer than 400,000, and industry and commerce was almost non-existent. Weizmann's oasis of learning gave priority to agricultural and medical research relevant to local needs.

Expansion of the institute began in 1944, when, at a party organized in New York to celebrate Weizmann's 70th birthday, he was asked by his wealthy friends what he would like as a present. "I need nothing for myself", he replied, "but if you wish, do something for the expansion of the institute." An international scientific committee was formed shortly after, and the multidisciplinary Weizmann Institute, with more than 60 laboratories, was opened in 1949.

Today, the institute has 1,500 staff, more than three-quarters of whom are scientists, engineers or technicians, including 200 tenured professors — between five and ten new ones are recruited annually. Since 1988, eight departments have been closed, six new ones opened, and two created by merging existing departments. "Research institutes thrive on change", says Harari.

Last year, its budget amounted to almost 350 million shekels, 40 per cent of which came from the government, almost one-third from donations, and one-third from research grants and contracts. Although spending on new facilities continues to outpace the renewed growth in available funds, the Weizmann has overcome the financial difficulties it experienced in the 1980s and has managed to balance its budget for each of the past four years. □

Israel plugs into EU research

ISRAEL is to sign a broad cooperative agreement with the European Union (EU) under which it will become eligible to compete within the EU's five-year Framework research programmes.

Because subscriptions are based on gross national product (GNP), Israel will pay just US\$30 million annually to take part in a programme that has a budget of ECU12.3 billion for the next five years. "We will pay according to GNP, but will win according to proposals. We therefore will do well", says one researcher confidently.

One official at the European Commission admits that Israel will be a "formidable competitor" within the programme, given its strong science base, its large existing network of international collaborations and its familiarity with seeking funds from organizations abroad.

Agreement between the EU and Israel was expected to have been reached several weeks ago, but has been delayed because of wider negotiations over trade issues. The ministry of industry and trade has also been seeking concessions from the commission to allow Israeli officials to sit on the committees that decide what funds are awarded and to whom. Israel may consequently miss the deadline for submitting some applications within the fourth Framework programme which runs from 1995 to 1999, says one researcher.

Joining Framework will "make a big difference to us", says Emanuel Marom, vice president and dean for research at Tel Aviv University. Similarly, the Weizmann Institute, which has just opened an EU liaison office in Brussels, "wholeheartedly" welcomed the move. □



Weizmann Institute, with Koffler accelerator of the Canadian Centre of Nuclear Physics in the background.

Schools face science overhaul

ISRAEL is to embark on an ambitious scheme, costing several hundred million US dollars, aimed at improving science and technology education throughout the country, following recommendations made last year by a review committee — the Superior Committee on Science, Mathematics and Technology Education — appointed in 1990 by Zevulun Hammer, the minister of education.

The recommendations of the committee — which was chaired by Haim Harari, president of the Weizmann Institute — are contained in a report — entitled *Tomorrow 1998*, 1998 being the 50th anniversary of the foundation of the state of Israel. The report calls for the construction of new teaching centres, retraining of teachers, redesigning of teaching materials and a complete reorganization of school syllabuses.

It says, for example, that a national programme should be established to improve studies in mathematics, the natural sciences and technology throughout the education system.

These areas should also become a required part of the syllabus from pre-school to high school, it recommends.

In a time of rapid technology change, the report argues that teaching of basic disciplines has become "particularly crucial". It identifies more mathematics teaching at all age-levels as a priority.

Laboratory experiments should be integrated throughout such courses, say the report, even if this requires extensive building of new facilities. Similarly, computing should be introduced throughout the school and teacher training systems, it says. The cost of the entire scheme is estimated at 300 million shekels annually over the first few years.

"We are now making a big effort to implement the report's recommendations, says Benjamin Geiger, dean of the Feinberg Graduate School at the Weizmann Institute, whose science teaching department has itself been involved in developing science curricula for Israeli high schools for almost thirty years. □

with metabolic resistance, which confers on the plant the ability to degrade the herbicide to non-toxic products, as most of the herbicide would be metabolized before it ever reached the roots. Instead, the researchers engineered the plant with target-site resistance, which involves modifying the plant enzyme targeted by the herbicide so that it no longer binds the herbicide.

By using three sorts of target-site resistances together, they controlled the broomrape parasite (see *Nature* 374, 220; 1995). The doubling in the yields of crop plants obtained as a result would more than offset the cost of transgenic seed and herbicide, says Gressel, who also points out that the approach should be used only with crops that do not interbreed with related weeds in the same locality.

But transgenic plants will provide only a stopgap. Each flower stalk produces 70,000–100,000 seeds, and the consequent high mutation rate means that it rapidly develops resistance to herbicides. Using computer models of resistance, the group has devised a management strategy which predicts that leaving 5 per cent of plants in the field susceptible to the weeds should slow the development of resistance from three years to between seven and ten.

Meanwhile, Gressel is studying biological control as a potentially more permanent solution. To do so he identifies healthy plants in infested fields, and isolates from them several hundred strains of fungi as candidates for mycoherbicides. The problem with mycoherbicides is that millions of spores need to be sprayed onto plants to overcome their defence systems and obtain infection. This is not economic, and to reduce the number of spores needed, Gressel is now carrying out basic research to find ways of disarming the plants' defences. □

Space on a shekel

NINETY-EIGHT per cent of Israelis write backwards, and the country's space agency is no exception. Whereas everybody else launches towards the east to hitch a ride on the eastward rotation of the Earth, Israel launches towards the west to avoid violating Arab airspace, and to ensure that launcher stages fall into the Mediterranean, and not Saddam's backyard.

Israel joined the small group of nations that build and launch satellites in 1988, when it put into orbit the 156-kg experimental satellite Ofeq-1. In 1990, it launched a clone, Ofeq-2, and earlier this year a second generation platform, Ofeq-3, widely thought to carry remote sensing equipment for spying.

The lightweight Ofeq platform is designed to be suitable for various scien-

Weed-killing at Weizmann

AGREEING to contribute an article to a multi-author treatise can end in tears. The publisher may be so obscure that its proof-readers may be the only people to read the text. Or a polite note may arrive, invariably after two years of silence, apologizing for the publisher being made bankrupt, and the book being shelved.

But Jonathan Gressel, from the Weizmann Institute's department of plant genetics, will not regret contributing an article to a book, published in the United Kingdom, outlining a potential control strategy for the parasitic weeds, broomrape and witchweed.



Jonathan Gressel.

A few weeks after the book was published, he received an invitation to a meeting with the Egyptian minister of agriculture, a professor of plant physiology and horticulture, and also Egypt's vice-prime minister. The outcome was a trilateral Egypt–USA–Israel programme, worth US\$3 million over four years.

Broomrape (*Orobanch* spp) and witchweed (*Striga* spp) are parasitic flowering plants that develop underground in the roots of the host plant. The parasite completes its life cycle by sending up a flowering shoot which "basically tells the

plant 'I screwed you,'" says Gressel.

In Africa, witchweed is estimated to infest 21 million hectares of grain-producing land, and cause 4.1 million tonnes of grain loss. Broomrape threatens 16 million hectares of cultivated land in the Mediterranean and West Asia, and according to Gressel, has caused Egypt to go from a grain exporter to a grain importer. The yields of infected plants are 30 to 70 per cent that of uninfected plants. "Looking at it another way, killing the parasite would double yields," says Gressel.

Given that no herbicides are available to kill the parasites selectively, chopping off flower stalks as soon as they appear remains the best means of control. The problem is that the parasites can lie dormant in the soil for up to ten years.

Gressel and his colleagues have been pursuing an alternative control strategy, aimed at producing crops resistant to particular herbicides. Treat the crop with the herbicide, and you should eliminate the parasite while leaving the crop plants untouched.

In designing a system, the group opted for a systemic herbicide that would be transported, like a sugar, through the plant's vascular system and reach the parasite in its roots. Directly applying herbicides to the underground parasite was considered impracticable.

This ruled out engineering the plants

tific and commercial missions, and is part of Israel's effort to develop space capabilities, and boost the competitiveness of its industries in international markets.

The launches are also a personal success for Aby Har-Even, director-general of the Israel Space Agency (ISA) since January, who headed the development of Israel's solid-fuel three-stage Shavit launcher. Har-Even joined the agency from Israel Aircraft Industries (IAI) after retiring from the army in 1979.

His agency, a five-man outfit located in the northern outskirts of Tel Aviv near the city's university campus, manages Israel's small but growing space programme. It controls a budget of only a few million dollars annually itself, but both proposes national priorities to the government, which spends US\$20–\$30 million on space every year, and coordinates approved projects.

ISA was created in 1983 from the National Committee for Space Research, set up by the Israel Academy of Sciences and Humanities. Although closely linked to the defence industries, it has focused on civilian goals, says Har-Even, because military links "complicate international collaboration".

Developing infrastructure for space activities in academic institutions and in the defence and electronics industries is ISA's main job, according to Har-Even. Indeed, some of Israel's most advanced space technologies are emerging from an unusual programme at the Asher Space Research Centre at the Technion Institute of Technology in Haifa.

Students there, with help from immigrant Russian scientists, have designed and built a sophisticated 50-kg satellite with a power consumption of just 20 watts. Techsat-1, as it is known, was developed for just US\$3.5 million, as almost three-quarters of the equipment was donated by aerospace companies keen to develop new ideas.

The bargain Techsat-1 was lost in March, however, during an equally bar-

gain, but failed, launch on a converted Russian SS-25 ballistic missile which was flying for the first time — the launch fee was just US\$250,000. Har-Even puts a brave face on the failure, pointing out that the Techsat structures that have been built up remain. Meanwhile, the government this month agreed to fund a replacement, Techsat-2, while Har-Even has not ruled out using the Russian launcher again.

The first satellite carried a simple camera that would have provided scientific and environmental data on forests, agriculture, coasts and urban areas. But El-Op Electro-optics, the Rehovot-based company that supplied the instrument, is hoping to fly a multispectral camera on Techsat-2, which could be used in addition for geological research and monitoring of water pollution.

ISA is also supporting TAUVEK (Tel Aviv University Ultra-violet Explorer), a US\$10-million cluster of three ultraviolet telescopes, which will be flown on the Russian-built Spectrum Roentgen-Gamma (SRG) international space observatory, scheduled to be launched by Russia next year, and which will also carry instruments from France, Italy, Denmark, Switzerland, the United States and the United Kingdom.

The future of Israel's fledgling space programme now lies in commercial exploitation and greater international collaboration, says Har-Even. Economic factors now drive "80 per cent" of the space programme, he says. The United States, for example, has agreed that Israel's Shavit can compete with the small Pegasus launcher in its domestic civilian market, provided half the industrial returns go to US companies.

Israel also plans to launch the first of two geostationary telecommunications satellites this year, as part of its main supposedly private venture, the US\$190-million Afro-Mediterranean Orbital System (AMOS), in which the European companies Alcatel Espace and Deutsche Aerospace have also invested.

But old habits die hard,

and the Israeli ministry of finance is said to have asked for subsidies of US\$100 million for AMOS as part of its plan to

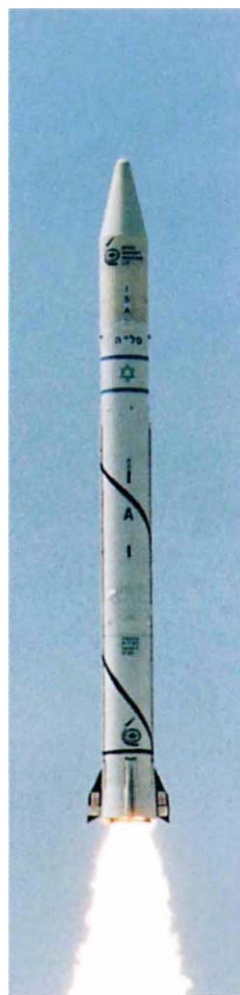
support the loss-making state-owned company Israel Aircraft Industries. Many also remain sceptical of AMOS's commercial viability in the Middle East television market, where many already subscribe to cable, or other satellite networks.

ISA's international cooperation is focusing on remote sensing, and last year the agency signed a US\$1-million three-year deal with the French space agency CNES covering research, data collection and technology. One result is the launch of the YAHTEL programme, a Hebrew acronym for the use of satellite imagery in remote sensing for geological, agricultural and hydrological applications, including measurement of ground salinity.

The deal has been soured somewhat, however, by a decision by SPOT-Image, the company that supplies French satellite images, not to wait for ISA programmes, but to authorize El-Op to provide data from SPOT satellites, whereas the International Centre for Technological Forecasting and Analysis at Tel Aviv University had previously been the exclusive distributor of SPOT images received by ISA's ground station.

ISA also intends to build a ground station to receive images from the ERS satellites operated by the European Space Agency (ESA). It is also collaborating with the Dutch space agency, under an ESA programme, SLOSHSAT. The experiment will study sloshing — which has apparently become a problem with the use of bigger fuel tanks in satellites — in a tank of water launched to 100 km, and juggled around by Israeli nitrogen boosters.

ISA's ultimate ambition is to become an associate member of ESA, says Har-Even, although discussions are at an "early stage". "We want to really cooperate", he says, pointing out that Israel could provide ESA with expertise in its proposed small satellite programmes. Joining ESA might also enable Israel to avoid launching towards the west — which reduces payload capacity by half — by swapping its launchpad at the Palmachim military base near Tel Aviv for ESA's facilities in Kourou, French Guiana. □



The solid-fuel Shavit launcher ascends.



Aby Har-Even, director general of the Israel Space Agency.

University on the up

ROCK music thumped around the 220-acre campus of Tel Aviv University (TAU) last month as students celebrated the end-of-year campus party that traditionally precedes the exams. Spirits were lower this time last year, when exams ran into the summer vacation, following a 77-day nationwide strike by academics over pay — TAU narrowly avoided losing an entire academic year because of the strike.

TAU, which is in the northern outskirts of Tel Aviv, was founded in 1956, and is now Israel's largest university. It has grown by a quarter over the past five years, and now employs 1,800 faculty and teaches 25,000 of Israel's 90,000 university students.

It has also recently opened the interdisciplinary Adams Super Centre for Brain Studies and the Porter Super Centre for Environmental and Ecological Studies. The university also houses two of the five centres of excellence set up last year by the Israel National Academy of Sciences — in astronomy and superconductivity. TAU boasts the only astronomical observatory in the Middle East.

TAU is also cooperating with the Weizmann Institute and Hebrew University to build two genome centres, one in

genetic diversity and the other in bioinformatics. Its main interests are genetic diseases that particularly affect Jewish populations, such as Gaucher's and Tay-Sachs. The university also holds a joint patent with Germany's Max Planck Institute on transgenic herbicide-resistant wheat.

Two-thirds of the university's budget comes from the Planning and Grants Committee (PGC). The US\$3 million set aside for research within the PGC grant was cancelled last year, however, to help pay for the wage settlements negotiated after the staff strike. A further quarter of TAU's budget comes from tuition fees, and the remainder from grants.

Research funds are scarce, says Emanuel Marom, vice president and dean for research and development at the university. TAU cannot afford large research projects such as those at the Weizmann or Technion, he says, and most researchers must make do with initial funding of US\$50,000–\$100,000.

TAU is also poorer than Hebrew University in Jerusalem, partly because it is younger and lacks the latter's large endowment, and partly because it needs to cover all disciplines, according to Marom, who adds that "we are growing fast, and catching up". □

War and peace

HEBREW University is one of the three academic institutes that were established by Zionists long before the state of Israel was itself created in 1948. The university opened in 1925 at the Mount Scopus Campus to the north of the Old City of Jerusalem, with 141 students, 33 faculty and research programmes in chemistry, microbiology and Jewish studies.

Werner Braun

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Hanoch Gutfreund, president of Hebrew University.

By 1947, the university had added departments in medicine, science, humanities, education and agriculture (in Rehovot), and had 200 staff and over 1,000 students. But the following year, the Mount Scopus Campus was cut off from the Israeli-held parts of Jerusalem during fighting in the war of independence.

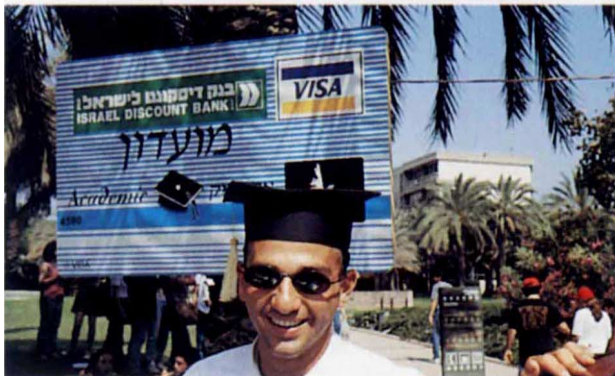
After operating from temporary facilities throughout the city for five years, the university built a new campus at Givat-Ram in the centre of the city, followed a few years later by a medical school campus at Ein Kerem in southwest Jerusalem.

After Israel recaptured Mount Scopus during the Six-Day War, the university rebuilt and expanded its previous campus there, and in 1981 this became its main campus again. Today, the university has over 23,000 full-time and 14,000 part-time students. Hebrew University also claims to carry out over 40 per cent of Israel's civilian research.

Last month, Hebrew University also agreed to set up an International School for Molecular Biology and Microbiology for Peace in association with the United Nations Educational Scientific and Cultural Organization (UNESCO). The school will bring together scientists from throughout the Middle East to help tackle regional health and environmental problems. □



An aerial view of the Tel Aviv University campus, on the outskirts of the city.



A student at Tel Aviv University campus party selling student loans. As most Israelis are in the army from the age of 18 to 20, students tend to be older than their counterparts elsewhere.

Science strikes back

THOUSANDS of school students and their teachers in Jerusalem are now using fully-equipped modern research laboratories in chemistry, physics, biology and computing, as part of a new scheme aimed at encouraging children to pursue science courses.

The Belmonte Science Laboratory Centre for Youth, as it is known, is the brainchild of Itzhak Parnas, the chairman of the High Committee for Strategic Planning (page 722), who is also a neurobiologist at Hebrew University. The centre, which was opened in 1990, is located at the university's Givat Ram campus in downtown Jerusalem.

The idea is to address the lack of good laboratory facilities in most high schools by creating one well-funded and maintained centre, serving all the schools in a given area — each class attends the centre several times per term. Students are taught by their own teachers, who themselves have been trained in the laboratory beforehand by PhD students from Hebrew University, who also supervise the laboratory experiments.

The centre's purpose is both to bring

teachers up to date with developments and techniques in scientific research, and to encourage more pupils to pursue science at school, and afterwards at university. "I don't think that all kids select humanities because they want to, it's partly



Itzhak Parnas, catching them young.

because we don't show them the glory of science," says Parnas, who directs the centre.

At present, however, the centre is only open to 11th and 12th graders — which corresponds to the last two years of school — whereas the decision to take science courses at this level is taken the year previously, in 10th grade. Parnas plans to expand the centre, however, to open it to 10th graders. Meanwhile, plans are also under way to create 18 similar centres throughout the country. □

Biotech drugs comes to market

ISRAEL'S drug industry seems poised for a boom. Beta-interferon, a drug that slows the progression of multiple sclerosis, and which is produced by Interpharm, is expected to be approved in Europe next year, and to reach annual sales of US\$100 million by the end of the decade.

Another multiple sclerosis drug, called Copaxone, being developed by Teva pharmaceuticals, is expected to reach the market next year, and analysts predict it will reach annual sales of US\$388 million by 1998. A genetically-engineered version of human growth hormone produced by Bio-Technology General (BTG) has also been approved in the United States.

Interpharm was created in 1979 to mass produce interferon- β for clinical trials. Ironically for Israel, the initial interferon is produced from foreskin fibroblasts. Interpharm, whose plant is near the Weizmann Institute in Rehovot, also teamed up with the Swiss company, Ares Serono, which now holds a majority stake in the Israeli company.

The technology for producing recombinant interferon- β was developed by Michel Revel, a researcher at the Weizmann Institute, who is also a director of the company. Revel was the first to produce beta-interferon in Chinese kidney hamster ovary cells (CHO), which yield a glycosylated molecule that is better tolerated by patients than interferon- β produced by conventional systems that using *E. coli*.

Interpharm's interferon- β has also been shown in early clinical trials to be an effective treatment for colon cancer (in combination with 5-fluorouracil) and small-celled lung cancer. "These are new applications that show the drug has a big future", says Revel. The company also produces interleukin-6, which might be used both to increase platelet counts in patients undergoing chemotherapy, and to prevent metastases of non-immunogenic tumours.

Two of BTG's founders also joined the company from the Weizmann Institute, where one had been a student of Revel's. Another of Revel's students founded Organics, a diagnostics firm renowned in particular for its HIV test kits.

Teva's multiple sclerosis drug Copaxone — which has been found to reduce the rate of relapse in patients — is also the product of research at the Weizmann Institute. The drug is the outcome of 25 years of research at the institute into copolymer-1 (COP-1), a synthetic protein designed to mimic basic protein, a major component of the myelin sheath, the tissue damaged in the exacerbating/remitting (ER) stage of the disease. COP-1 was first proposed by Michel Sela and Ruth Amon, both from the institute, as a tool to

Swords into ploughshares

ISRAEL'S ministry of industry and trade (MIT) will this year pay out more than US\$450 million in subsidies for industrial research, US\$150 million more than last year, when it sponsored more than 1,300 programmes and 800 companies. "The rate of participation is enormous", says Shuki Gleitman, MIT's chief scientist, "I would use the word 'boom'".

MIT provides companies with up to half the costs of research projects, most of which are near-market. If a project succeeds, the company must reimburse the sum in royalties. If it fails, it pays back nothing. This year MIT will collect US\$60 million in royalties, according to Gleitman.

Another programme, MAGNET, is aimed at encouraging companies to collaborate on 'pre-competitive' generic research. The programme, which has been running for just over two years, provides companies with up to two-thirds of the costs of a project. To qualify for funding, consortia must also include at least one academic institution.

Israel exported US\$11.5 billion of goods last year, and more than half of these were produced by technology-based companies. Thirty years ago half Israel's exports were agricultural produce.

The electronics industry accounts for most technology exports and almost three-quarters of industrial research and development in Israel. The industry was created from scratch by massive government investment in the defence industries, following the decision by France to impose an arms embargo after the Six-Day War in 1967.

While sales of military equipment have remained flat over the past few years, those of civilian electronic goods have been increasing by around 20 per cent annually. Much of Israel's defence industry focuses on software and telecommunications equipment which have applications in civilian sectors. Last year civilian goods represented four-fifths of electronic exports compared with just half in 1988. □

study how T-cells recognize and destroy myelin.

But while Weizmann institute research is driving some of Israel's most successful biotechnology companies, the attitude of academics at the institute and Israeli universities is discouraging commercial development of basic research, according to some researchers. In particular, scientists who become involved in commercialising their research are alleged to suffer diminished prospects for promotion. "It's academic suicide", says one researcher.

Haim Harari, the president of the Weizmann, admits that this is a problem. "I support technology transfer", he says, "but those involved do pay sometimes in terms of their careers." The faculty committees that decide promotions often frown on industrial ties, he says: "there is a contradiction between the policy of the management and that of the majority of the professors, who democratically elect the committees."

While finding venture capital for biotechnology start-ups is fairly easy in Israel, some observers are also concerned that such start-ups lack the regulatory and marketing expertise to be able to survive in the international market. "We have had 60 start-ups in biotech over the last few years, but most will not make it," predicts one scientist. Israeli companies need to hook themselves onto multinationals, argues Revel, if they are to sell internationally. "Growing big by yourself is the only alternative", he says, "and this takes too long." □

In the shadow of Ben Gurion

TWENTY-thousand chickens suffocated last month, as temperatures climbed into the upper-forties during Israel's worst heat wave for 40 years. But temperatures inside the main building of the Jacob Blaustein Institute for Desert Research in Sede Boker, in the Negev Desert, nonetheless remained comfortable — without any assistance from air-conditioning.

The building provides a balanced temperature, day and night, by exploiting appropriate architectural design concepts, many of which were known to ancient desert settlers, but have been forgotten in the design of many modern buildings. Embedding a massive stone column, or 'heat sink', in the centre of a building, for example, dampens excessive temperature fluctuations by absorbing and storing large amounts of heat.

Such architectural designs are also symbolic of a profound shift in the goals of Israeli desert research, away from conquering

the harsh environment, and towards living with the conditions it imposes. This shift is important, because desert research in Israel, besides its academic interest, is a key to the country's future.

The reason is simply explained. The desert area south of Beersheva account for two-thirds of Israel's surface, but less than a tenth of its population. The population density in the rest of Israel exceeds 500 per square kilometre, or half as high again as that of Japan or Holland, and if population growth continues at its present rate, it will be 2.5 times higher than that of either country within 30 years. These people will have to go somewhere, and the desert is one of the few areas available.

David Ben-Gurion, Israel's first prime minister, recognized the imperative of exploiting the desert, from the moment of Israel's creation. Indeed, he set an example by moving to the kibbutz at Sede Boker in 1953 at the age of 67, where he lived with few possessions, apart from his vast collection of books.

The research centre, which was established in 1974, is a faculty of the university that is named after him, the Ben-Gurion University in Beersheva, which was itself founded in 1969.

Ben-Gurion and his wife are buried on a ridge above the research centre, overlooking both the awesome canyons of the Zin Valley (shown on page 729), and the shops and houses of the Midreshed community which houses both Ben-Gurion's archives, and the 150 or so families who work either at the centre, or at the community's renowned boarding school.

But while Ben-Gurion's Zionist ideals of creating an "oasis of learning, an Oxford, in the desert" are perpetuated by the community, most of whose residents are either researchers or teachers — around 40 of whom religiously attend the weekly 'journal club' — his vision of making the Negev "bloom" have never really materialized.

"We've had to twist Ben-Gurion's philosophy," says Berry Pinshow a zoologist at the centre, whose wife is the curator of



Inscription by David Ben-Gurion, Israel's first prime minister, celebrates the creation of the Desert Centre.

the Ben Gurion archives at Sede Boker. "He wanted to fight the desert, but our philosophy is 'wise settlement' of the desert."

Earlier attitudes are captured by a zealous Zionist folk song, which roughly translates as "Oh dear homeland, we will cover you in roads and concrete". The homeland will be happy to hear that

Science in the sand

BERRY Pinshow, a zoologist, is one of the many fundamental researchers at the Jacob Blaustein Research Centre in Sede Boker who study the desert for its own sake. Indeed, like many at Sede Boker he is a desert enthusiast: "I love living in the desert, and the weather is terrific," he says.

His early career took an unexpected turn, however, when he obtained a postdoctoral position with a research group at Duke University in the United States that was renowned for its work on deserts. On arriving, he discovered that the laboratory had long ceased work on desert animals, and he ended up working in Antarctica for several years on the ecology of emperor penguins.

One area in which the now thawed-out Pinshow works is on the physiology of migrating birds. Israel is a good place for such studies as between one-tenth and one-fifth of all birds migrating to Africa pass over the country, which lies along one of the three main north-south migrating routes.

In particular, Pinshow is studying the role of water balance and plasma volume during migration. Apart from field studies, he is also using Tipler homing pigeons — which can fly for up to five hours at speeds of up to 60 km an hour — as an experimental model. The birds have a genetic defect that makes them fly in large circles, and they can be trained to return to the laboratory on hearing a loud whistle. □



Closed culture of *Spirulina*.

today's visionaries prefer to adorn her with solar energy panels, harnessing the desert's ample sunshine, integrated fruit and fish farms using the plentiful, but brackish, water that has accumulated in aquifers below the Negev, and plants engineered to flourish in her spartan soil.

Indeed, while efforts to produce more fresh water may someday provide sufficient amounts for large-scale irrigation of the desert, Samuel Applebaum is now concentrating on developing integrated fish farms at the centre that could exploit the enormous quantities of warm 'fossil' water lying a few hundred metres below the Negev.

This pollution-free water, he points out, is ideal for cultivating highly productive species such as *Tilapia*, catfish and eels, while the excrement-rich waste water can later be used to irrigate salt-resistant crops such as tomatoes and melons.

Because *Tilapia* has scales and fins — and therefore qualifies as kosher — it sells well domestically, where it is known as St Peter's fish. Eels, which are cultured in Sede Boker at such high densities (up to 200 kg m⁻³) that it is difficult to see the water for flesh, are definitely not Kosher, and are exported as delicacies. Ornamental fish, which are also farmed at Sede Boker, also account for \$9 billion of Israel's annual exports.

Avigad Vonshak is also pursuing aquaculture, but this time of algae. Earlier ideas of producing algae for cheap protein have been abandoned, however, and Vonshak is exploring genetic techniques and closed-cultivation systems to grow



Negev Desert, from the research centre.

algae which are either expensive themselves — such as *Spirulina*, which sells as a health food — or can be used to produce high-value chemicals.

Avi Golan is working on the biochemistry of another cash crop, this time pistachio trees, which can flourish with almost no water. Golan has recently won a grant from the European Union, along with colleagues in Egypt and Belgium, to establish a germplasm bank and breeding programme for the tree. World demand for pistachio nuts outstrips supply.

Golan is also carrying out an ethnobotanical study of desert plants to find compounds with properties of interest to the biotechnological industry. But his efforts to compile local folk knowledge of medicinal plants before this is lost have been frustrated by his discovery that even the eldest of Bedouin nomads now seem to prefer the local oasis pharmacy to traditional medicines.

But despite the diversity of desert research at the centre, which has an annual budget of US\$10 million, one leaves Sede Boker with the uncomfortable impression that its vision of colonizing the desert may, like that of Ben-Gurion's before, fall short of its ambitions.

A prototype high-technology greenhouse in which plants need only about 10 per cent of their normal water requirements — which was described in *Nature*'s 1987 survey of Israel (see *Nature* 327, 583; 1987) — for example, remains just that, a prototype. Indeed, what the centre seems to lack are means of developing and commercializing the technologies it produces.

"Our impact is not as much as we would like it to be," admits Reuven Kopel, who manages the centre and is

also responsible for development of the greenhouse. "It's a process of educating government agencies to provide more funding", he says philosophically, "and that takes time." "The problem", agrees Yair Zarmi, deputy director of the centre, "is that the research groups are too small, and lack the critical mass needed."

Indeed, Israel Dostrovsky, who leads solar energy research at the Weizmann Institute, says that while the desert centre has developed impressive solar power systems, "time is against it". The centre is "too small and too isolated", he says, predicting that the much larger investments made by the Weizmann in solar power will allow it to produce commercial-scale systems long before Sede Boker does. □

A prophet in his own land

ISRAEL could soon appear on satellite images as one long strip of aluminium foil. That is, if Israel Dostrovsky, who heads the solar energy programme at the Weizmann Institute, gets his way. Dostrovsky is convinced that Israel will one day "go solar".

Israeli law already requires all buildings more than six storeys high to be equipped with solar panels for water heating, a move that has reduced the national electricity bill by 3 per cent.

But Dostrovsky, a former president of the Weizmann Institute, has much bigger ideas. "We are talking about a national system, we are talking about replacing oil", he says. Indeed, what sets Dostrovsky's research apart from much other solar research is that it is aimed at overcoming the main bottlenecks to developing systems capable of supplying whole countries.

Operating such large systems would mean, for example, that the energy collected would have to be stored and transported somehow. The obvious reason is that the sun does not shine at night — even in Israel, which averages more than 300 days of sunshine per year — or through clouds. Another is that while desert areas are the obvious site for the vast solar plants needed, these are far from population centres, where most electricity is consumed.

Another problem is that solar power installations are expensive, because they require large areas of collectors — a 1 m² collector can at best collect 1,000 kW per day. To compensate for this, conversion of the energy collected into electricity must be highly efficient.

Dostrovsky believes that high temperatures are the key to the problems of both storage and transport, and efficiency. Whereas most solar energy systems operate at low temperatures, the 64 parabolic mirrors and solar tower of the



Solar panels amid the domes, Jerusalem.



Solar Tower, The Canadian Institute for Energy and Applied Research, at Weizmann.

US\$15-million Central Receiver experimental facility at the Weizmann Institute (see below) can generate temperatures of up to 2,000 °C, although it usually is operated at around 1,000 °C.

To solve the problem of storage, Moshe Levy, another Weizmann scientist, has developed a catalytic process that uses solar heat to react methane with either steam or carbon dioxide at 900 °C to give a stable mixture of hydrogen and carbon monoxide (synthesis gas). This reaction is endothermic, and all the solar energy put in is contained in the synthesis gas.

This synthesis gas can be easily stored at high pressure and transported, and the energy recovered when needed simply by reversing the reaction using an appropriate catalyst — this also regenerates the starting products. The theoretical efficiency of the process is 100 per cent, and Levy has obtained efficiencies as high as 80 per cent in practice.

Dostrovsky says his group is now testing this regenerative system within a 0.5-MW solar power plant, and is negotiating to build a 10-MW plant. "It would be nice to build this in Israel," says Dostrovsky, "but we will go wherever the funding is."

The temperatures produced in the Weizmann system are also similar to, or higher than, those in industrial power cycles. Indeed, while conventional power stations burning fossil fuels, which operate at around 500 °C, are around 35 per cent efficient, the conversion of energy to electricity at 1,000 °C can in theory be as high as 60 per cent.

Such levels of efficiency are reached in the latest generations of power stations. These use a process that involves first driving hot (1,200 °C) compressed gas produced by combustion of fuel through a gas turbine — which works in much the same way as a jet engine — and then exhausting it through a conventional turbine at 500 °C, in what is known as a 'combined cycle' system.

Over the past five years, the Weizmann group — in collaboration with the Israel Electric Corporation and Ormat Turbines — has been testing a 50-kW gas

turbine system in which the hot compressed air used to drive the turbine is produced instead by first passing air through long ceramic tubes that are heated directly by solar radiation. The group also has plans to scale up to a 500-kW plant, although the development costs of anything larger would be prohibitive and would require commercial investment.

Dostrovsky says he is now "more optimistic" about the prospects for solar power. In particular, he predicts that Israel's growing thirst for water, rather than its demand for electricity, is making a national solar power system a more attractive proposition.

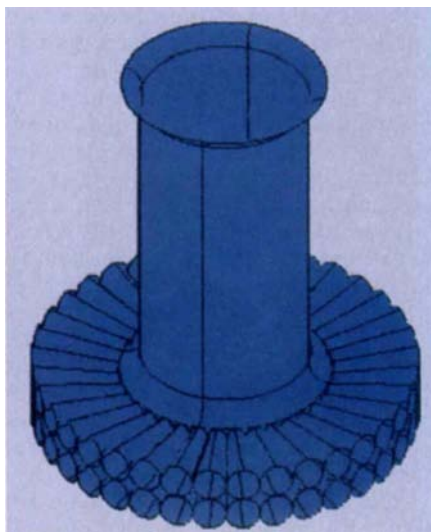
Desalination of sea water is the ultimate solution to the scarcity of water in the Middle East, argues Dostrovsky, but while the technology required is mature, desalination requires huge amounts of cheap energy to make it economically viable. Combined solar energy/desalination plants, producing both electricity and water will be the best solution, he predicts. □

Tower of Babel

IN 1988, rain fell from a high cloud onto the desert city of Beersheva, cooling the warm and dry column of air below, and causing the colder air to collapse towards the cacti at high speed. The suddenly air-conditioned local airport suffered US\$1 million of damage.

Researchers at the Technion Institute of Technology in Haifa are now trying to artificially create similar conditions to produce continuous wind, which could be converted into copious amounts of cheap electricity.

Both the plan and the physics are straightforward. Find a hot and dry district.



Schematic shape of energy towers.

Erect a hollow tower at least 1 km high, or around three times taller than the Eiffel Tower. Pump tonnes of sea water to the top, and atomize it. Evaporation should then cool the surrounding warm and dry air, and cause it to tumble down the tower at around 60 km per hour. Then, simply slot in a wind turbine at the bottom, and plug it into the national grid.

The idea is not new — it was first proposed by Philip Carlson in the United States in 1975 — but little serious technological development was done until the Technion group took up the project 13 years ago. Over the past four years, the ministry of industry and trade has generously funded the project.

On paper, energy towers, as they are called, appear a solution to Israel's power needs, and indeed the world's, as in theory they could supply many times the current world energy needs, at a cost as low as 0.7 cents per kilowatt hour. Global circulation produces plenty of the hot air needed.

The political problem is that the same straightforward physics also predicts that the power of energy towers increases dramatically with the height and diameter of the chimney. While the government is said to be about to invest US\$30 million in the building of a 50-MW 200-metre prototype, sceptics argue that this would test only the basic principles. Proving that the process is economic, they argue, will require gambling nothing less than US\$650 million on the real thing, a chimney at least 900 metres high.

Deciding whether to take such a gamble is a political dilemma. Moreover, as any engineer knows, extrapolating energy towers directly from the drawing board to the building site is asking for unpredictable problems, on top of the predictable perils such as salt troubles in one's tower and turbines. Such snags could yet throw cold water on the cleverest economic calculations. □

War of the wells

WATER supplies in the Middle East are like a blanket that is too small. If one country consumes more, less is available for its neighbours. Indeed, Avraham Sela, a political scientist at Hebrew University in Jerusalem, has argued that water could cause war in the region, in particular between Syria, Iraq and Turkey, who compete for the waters of the Tigris and the Euphrates.

Israel also competes with Syria and Jordan for water, while Gaza depends on Israel for its supplies. Middle Eastern countries met this month in Amman, Jordan, for the latest in a series of multilateral negotiations about water. Israelis, Palestinians and Jordanians are also considering drafting a 'water charter' among themselves to improve the manage-



Map showing alternative alignments for the Dead Sea project's canals.

ment of supplies.

The Sea of Galilee supplies one quarter of Israel's fresh water, and ground water the rest. But Israel's water consumption, 1.8 billion cubic metres (BCM) already outstrips its renewable freshwater resources, 1.5 BCM, the difference being made up, for example, by reusing treated waste water. Drinking water supplies are being threatened further by pollution of ground water.

To ease its water shortages, Israel is reassessing a proposal for an ambitious engineering project — The Dead Sea Hydro Project — that would involve constructing canals from the Red or Mediterranean Seas to the Dead Sea. The project, as originally envisaged, was aimed at exploiting the 400-metre difference in height between sea level and the Dead Sea, to produce electricity.

But the project, which was rejected by the energy ministry in 1986, has now been revived, with the major emphasis not on electricity production but on desalination. Its main aim is now to produce 0.8 BCM of water annually for Jordan and Israel using a hydrostatically powered reverse-osmosis desalination system.

The energy would be created by discharging 1.2 BCM of water in the Dead Sea annually, a volume equivalent to the sea's annual deficit. Three canal routes are currently being studied (see map). The total cost of the project is estimated at US\$5–\$7 billion, depending on the route, and would solve water problems in the region for 20 to 30 years. Referring to the proposals, Ezer Weizmann, Israel's president, has said that "a big project is needed to symbolize peace, and show that something is being done". □

Cool crops

BEING a plant in a hot and arid area can be tough. Just when it breathes relief at having survived a heat wave and a long drought, along can come a period of particularly scorching sun, throwing it biochemistry into a spin, and causing the release of toxic oxygen molecules that damage and eventually destroy its photosynthetic apparatus.

Help for such sun-sensitive plants may be on its way. Ada Zamir's group at the Weizmann Institute, have recently worked out the mechanism by which the sturdy saltwater alga *Dunaliella*, the Land Rover of the algal world, survives scorching sunlight. The alga flourishes in the brackish marshes of the Sinai desert, and even in the Dead Sea.

Zamir's group have discovered a protein, known as Cbr, that *Dunaliella* synthesizes when its photosynthetic system is threatened by sunlight, and cloned the gene coding for it. It has also found evidence that the protein binds with the carotenoid pigment zeaxanthin, which is also formed under conditions of extreme sunlight — in a move which diverts excessive sunlight energy away from the photosynthetic apparatus.

The findings raise the possibility that weakling crops might be engineered into hardy desert survivors better able to resist the strong sunlight in such hot and arid areas. □

Nothing but "Perrier"

THE Dead Sea is already the lowest lake in the world — around 400 metres below sea level — and since 1960 its level has been dropping by 0.5 metres annually. The main cause is increased use of water from the Jordan river, which flows into the Dead Sea, while rain in the region only amounts to around 60 mm annually.

A dropping Dead Sea is bad news both for tourism and for the operation of the Jordanian and Israeli Dead Sea works. But the new salty shores that have been exposed as a result of the drop in water level have provided a unique opportunity to study the colonization of hostile environments.

Indeed, some desert plants have already invaded these shores, and become established. This in itself is fairly remarkable, given that the salt concentrations in the soil shore are three times those of ocean water, but what is even more surprising is the particular way in which the plants — such as species of *Sueda*, *Anabasis*, *Atriplex*, *Hamada* and *Tamarix* — have managed to survive.

An analysis of the isotopic composition of the water in the plants — carried out by Dan Yakir of the Weizmann Institute, and Yoseph Yechieli of the Geologi-

cal Survey of Israel (see *Nature* 374, 803; 1995) — showed that it differed from that of the salty soil water. Instead, the isotopic fingerprint matched that of the sporadic winter floods that flow into the area from the Judean hills.

This suggests that the plants are able to discriminate between the chemical differences in flood water and that of the surrounding salty soil. "Such a capacity", the researchers point out, "may be a prerequisite for successful invasion of highly stressful environments, allowing for the early onset of terrestrial biospheric activity".

Such experiments may come to an end, however, if a project aimed at exploiting the 400 metre difference between sea level and that of the Dead Sea to generate electricity (see figure) goes ahead. The plan which would involve constructing canals to allow water to flow from the Mediterranean or Red Seas into the Dead Sea would restore its historical level. □

Power in parallel

A SUPERCOMPUTER is the "first priority" on the shopping list of the ministry of science and arts (MOSA), according to Itzhak Parnas, the chairman of the ministry's High Committee for Strategic Planning. Israel has previously been prevented from buying one by a ban by the United States on the export of supercomputers, said to have been imposed because of Israel's refusal to sign the Nuclear Non-Proliferation Treaty.

But President Bill Clinton has now promised Yitzhak Rabin, Israel's prime minister, that he will lift the restriction, says Parnas, who adds that MOSA is coordinating the pooling of US\$20 million from various Israeli organizations to buy the country's first machine. Responsibility for operating the machine will then be passed onto the Inter-University Centre for Computation.

Faced with the ban, however, Amnon Barak, a computer scientist at Hebrew University, has improvised and developed a powerful supercomputer substitute based on linking 28 Pentium PCs using a modified UNIX operating system. This parallel computing system, which cost just US\$150,000 to build, distributes parallel tasks among the PCs, allowing them to carry out calculations simultaneously. In particular, the software he has developed ensures that all computers have the same load at all times. □

Fertile discussions

THE modern techniques of medically-assisted procreation pose a dilemma for orthodox Jews, because while they are concerned about the ethical issues these raise, they are keen to reconcile such technologies to their religious laws which oblige Jews to reproduce.

Indeed, fertility is a major issue for orthodox Jews. This is reflected in the fact that while obtaining a divorce is generally difficult under Jewish law, a man can divorce his wife if she does not produce offspring within ten years.

At a recent conference on bioethics at Tel Aviv University (TAU), it was disclosed that secret talks on medical issues, and in particular the treatment of infertility, have been going on for over two years between four leading rabbis of the 'Haredi' (ultra-orthodox) community, and scientists, philosophers and lawyers.

Lord Immanuel Jakobovits, who is a former chief rabbi of the British Commonwealth, an orthodox expert in Jewish medical ethics, and one of the main architects of British government policy in bioethics, described the talks themselves as a "historic event without precedence" and the TAU conference as a useful attempt to "bridge the divide" between the scientific and ultra-orthodox communities.

Jakovovits told the conference that orthodox Judaism supports new technologies which treat infertility, given that the decreasing size of the Jewish population threatened the long-term survival of the Jewish population. He also approved the use of surplus human embryos for scientific research.

Research in infertility is also the focus of the Male Fertility Clinic at Bar-Ilan University. But whereas fertility has often been mainly studied by gynaecologists, urologists and endocrinologists, Binyamin Bartoov, a researcher at the university, is trying to develop a research discipline dedicated to the study of sperm.

As well as gathering epidemiological data, his team is developing techniques such as cell manipulation and selection, to allow sperm production from males who were previously considered infertile. All of the research is supervised by the *halakhah*, the leading decision makers in Jewish law.

But bioethics is only the latest example of where orthodox Judaism and science can clash. Although Israel is not formally a theocracy, state structures conform to orthodox Judaism, and religious parties still hold considerable influence in the parliament, or Knesset, where no party has ever held a majority and horse trading to form coalition governments is the rule (see page 718).

Occasionally, this political situation

does lead to major confrontations. Almost all the human skeletons from ancient populations which were previously housed at Israel's Antiquities Authority, for example, have recently been transferred to the Religious Affairs Ministry to eventually to be buried anonymously. Orthodox Jews had argued that research on such bones contradicted with Jewish laws forbidding the disturbance of Jewish graveyards.

But most potential conflicts between science and religion are sorted out amicably, as are many other conflicts between the law and modern everyday life. Lighting fires is prohibited on the Sabbath, for example, and ultra-orthodox consider this to include switching on domestic lighting. As a result, many homes are equipped with lighting systems which switch on automatically at dusk — some poorer households, which cannot afford the equipment make do in the dark.

Similarly, answering the telephone on the Sabbath is prohibited, because this is considered as "work" under Jewish law. This has resulted in the development of telephones which answer automatically, and place the caller on hold. As the connection is already established, picking up the handset does not then constitute work. □

Jurassic parchment

MAPPING the human genome depends on first breaking it up into manageable sized-chunks and then rearranging these chunks in the right order by identifying overlapping sequences. Geneticists in the United States and Israel are now using DNA techniques to solve another jigsaw — piecing together thousands of fragments from the Dead Sea Scrolls.

The scrolls, the oldest surviving copies of biblical texts, were discovered in 1947. Since then, around 15 of the scrolls have been translated, but analysing the remaining parchment is about as easy as making sense of a copy of *Nature* that has been fed through an office shredder, as it exists only as fragments — over 10,000 of them — in various states of decay.

The scrolls were written on animal skin, however, and still contain DNA, more than 2,000 years after they were written. Over the past year, researchers at the Hebrew University in Jerusalem and their colleagues at Brigham Young University in Utah, have been trying to piece together fragments by identifying which came from the same species, or even the same individual.

Most of the scraps of the scrolls seem to have been written on goatskin, but also on skin from sheep, ibex, or gazelles. Biblical scholars, have enthusiastically welcomed the research, because by showing which fragments belong together, it allows them to replace one enormous jigsaw with several smaller ones. □

The Bible enters the computer age

SCIENCE and religion are coming together at Bar-Ilan University in Ramat Gan, in a project that is combining computers and classical biblical scholarship to produce a definitive edition of the Bible, and its *mas-sorah* and *targum* commentaries, known as the *Mikraot Gedelot*.

The original version was published by Ya'acov Ben-Haim in Venice in 1525, who brought the then modern printing technologies to bear on the various hand-written manuscripts available. The new work has been carried out by Menachem Cohen, who holds Bar-Ilan's Weiser Chair in Medieval Biblical Commentary, in cooperation with computer scientists.

The revised *Mikraot Gedelot* is based on the *Aleppo Codex*, which is considered to be the most accurate mediaeval text available, in terms of usage and spelling. The team has already published three of the 18 planned volumes, and a CD-ROM version is under way.

With comparison at the touch of a button of not only passages, but also sentences and words, academic study of the bible will never be the same again. The world's best selling book is moving into the world of multimedia.

It is appropriate that such a research project should be carried out at Bar-Ilan. The university, which this year celebrates its fiftieth anniversary, is mainly dedicated to Jewish studies. All students must take a minor in Jewish history, literature, ethics and culture.

Apart from Jewish studies, the university has four other faculties: natural sciences — which covers most area of science — social sciences, humanities and law. Between 1955 and this year, student numbers at Bar-Ilan University have grown from 56 to 19,510, and staff from 24 to 1,306. Over the next decade, the university plans to double the surface of its buildings in a 70-acre extension to the campus.

Another peculiar analysis of the Bible that has been pursued by computer studies is that of Moshe Zeldman, a mathematician and rabbi in Jerusalem. As evidence of the Bible's divine origin, Zeldman has claimed to show that groups of words hidden in the text relate to events that occurred long after it was written.

His system, which studies equidistant letter sequences (ELS), has been pursued by a group of US and Israeli mathematicians and Rabbis, in particular Eliyahu Rips of Hebrew University. One computer search, for example, found ELSs corresponding to the Hebrew transliterations of Hitler, Germany, and Auschwitz, in the passages of Genesis dealing with another Holocaust, Noah and the flood. Coincidence? □

The ultimate in atom manipulation

A remarkable experiment carried out in Paris has shown that it is possible to change the phase of quantum matter waves by modulating a virtual mirror reflecting atoms with a radio-frequency signal.

MANIPULATING atoms and molecules individually is more than a mere sport, of course, but it is increasingly full of fun. In the past year or so, we have had so many demonstrations of interference between atomic beams behaving for all the world as if they were photons that it can be only a matter of time before somebody uses such beams to take a photograph of some previously inaccessible object, probably one with dimensions of a few micrometres. But the important benefits will come in other ways, notably in spectroscopy and further improvement of atomic clocks.

Now a group at the Laboratoire Kastler Brossel (a joint operation of the Ecole Normale and the Pierre and Marie Curie University in Paris, supported by the CNRS) has gone one step further, managing to modulate the phase of the de Broglie waves that represent the physical motion of atoms in circumstances where quantum rules apply. This, in simple language, is what the group has done.

Caesium atoms are dropped from a height of 3.3 mm onto what can only be described as a virtual mirror — the pattern of oscillating electrical fields external to a polished quartz prism within which a laser beam is totally internally reflected. The mirror is made to alternate between reflection and transparency with a frequency in the MHz region. The result is that the kinetic energy of the reflected atoms is increased or decreased by the equivalent in energy of an integral multiple of the modulation frequency. So much is demonstrated by letting the reflected atoms bounce up to the top of a ballistic trajectory before measuring the positions at which they come down again with a laser probe.

That account is deliberately and misleadingly artless. In reality, what A. Steane, P. Szriftgiser, P. Desbiolles and J. Dalibard describe is technical wizardry of the highest order (*Phys. Rev. Lett.* **74**, 4972–4975; 1995). Among other things, they laconically describe how they are able to transfer cold caesium atoms from one cold trap to another simply by letting them fall there under gravity.

Cold trapping is so much the essence of the game that one would be well advised to learn the acronym MOT (for ‘magneto-optical trap’). There is an excellently clear description of how such a trap functions, together with a diagram, in a News and Views article by Christopher Foot a few weeks ago (*Nature* **375**, 447–448; 8 June 1995). Briefly, a pair of coils set symmetrically

facing each other and carrying equal currents in opposite directions make a quadrupole magnetic field with a trapping point at the centre of symmetry.

That will trap paramagnetic atoms from the surroundings when the current is switched on. Cooling requires the optical part of the MOT, which is an array of six identical lasers pointing at the symmetrical centre and operating at a frequency near some optically resonant frequency of the atom, the 862 nm line of caesium, for example.

Steane and colleagues operate the uppermost of their two MOTs on a brisk duty cycle of 1.4 seconds. They collect 300 million caesium atoms in a second, cool them to 5 K by detuning the lasers by up to 9 half-widths of the atomic resonance (which creates the sea of ‘optical molasses’ in which atoms appear to be moving through an exceedingly viscous liquid). Then the lasers are detuned still further, allowing the atoms to fall 70 cm under gravity to a second MOT, operating on a different cycle. A second MOT is necessary because it must operate in a better vacuum than the first, whose surroundings must contain enough caesium for a trapful to be collected in a reasonable time.

That is where the fun begins. The essence of the experiment is the virtual mirror, which is a prism containing an internally reflected and linearly polarized beam (at the same resonant caesium frequency). The polarization of the beam is in the plane of the horizontal surface of the prism onto which the caesium atoms fall perpendicularly. The result is an intense electric field (oscillating at the frequency of the caesium line) above the reflecting surface and decaying with a scale height estimated at 0.19 μm . The result of that is to bounce the caesium atoms vertically backwards.

Apparently the virtual reflector works best when the operative surface is concave, making the effective reflector of caesium atoms a spot with diameter 0.4 mm. The laser beam that creates this field is the beam that must eventually be modulated at a MHz frequency.

With that arrangement, the function of the second MOT is first to compress and then to cool the 20 per cent of each load of atoms from the first trap that find their way into the second, and then to drip them onto the reflector. The next step is to select atoms with a particular velocity, which is done by the simple device of choosing the

interval of time between a first unmodulated and a second modulated laser pulse in the reflecting prism.

The time required for a caesium atom to move vertically upwards and then down again is evidently a measure of its initial velocity, which must be $2gt$, where g is the acceleration due to gravity (at Paris) and t the time. This first report of the experiment describes the results of measurements when the first unmodulated laser pulse (0.4 ms long) is followed after an interval of 52 ms by a second modulated pulse of the same duration; that corresponds to a velocity of 25 cm s^{-1} at the reflecting spot.

So what happens when, for the second pulse, the laser beam in the prism is modulated? At this point, there is a bunch of atoms falling downwards onto the reflecting spot at 25 cm s^{-1} (with a spread of less than 1 per cent), but they are bounced back with the same velocity. But on this occasion, on what is their third descent, the atoms are detected individually by their absorption of a probe beam 1.7 mm above the virtual mirror. This is a time-of-flight measurement, but the probe laser must be switched on at just the time that the infall of caesium atoms is expected.

The results are remarkable. If the second ‘modulated’ pulse is, in reality, unmodulated, the outcome is a simple gaussian distribution (which among other things makes it possible to tell exactly where the probe laser beam is located). But if the second pulse is modulated at 950 kHz, there is a central peak in the same location and two sidebands symmetrically placed on either side. What this implies is that the frequency of the modulation has been transferred to the kinetic energy of at least some of the caesium atoms, or that the phase of the matter (de Broglie) waves has been shifted by external intervention.

The question naturally arises, as always on occasions like this, of what devices will eventually be spawned by this experiment. The authors themselves are largely silent on the subject, although it is natural that they should offer their arrangement as a model for a natural beam-splitter of coherent atomic beams. But they also suggest that it may be a way of measuring g more accurately than is possible with macroscopic measurements, arranging for several unmodulated bounces before a final measurement. That would be worthwhile for its own sake, but somebody will think of something better.

John Maddox

Missense on the membrane

Dennis J. Selkoe

EMERGING from relative obscurity only two decades ago, Alzheimer's disease (AD) has captured the attention of both the scientific and general public as have few other diseases. This genetically and phenotypically complex syndrome of progressive cognitive failure occurs in different races and ethnic groups worldwide, and is the most common cause of late-life dementia in developed nations. All cases of AD develop countless extracellular deposits (plaques) of the 40–42-residue amyloid β -protein ($A\beta$) in the brain, and almost all also have many neurofibrillary tangles, intraneuronal bundles of abnormal filaments composed of highly phosphorylated forms of the microtubule-associated protein tau. The steady march towards deciphering AD takes another step forward with the discovery by Sherrington *et al.*, reported on page 754 of this issue¹, of a gene on chromosome 14 that appears to be responsible for a majority of early-onset (under 65 years old) cases of familial AD.

Although it is frequently stated that the cause of AD is unknown, geneticists have previously identified two genes, one on chromosome 21² and one on chromosome 19³, that are very strongly linked to the development, respectively, of early- and late-onset forms of AD.

Mutations

On chromosome 21, several missense mutations in the β -amyloid-precursor protein (β APP) gene have been found in a few families experiencing onset of AD in their fifties. All of these disease-causing mutations have now been modelled in transfected or primary cultured cells and shown to lead to altered proteolytic processing of β APP in a way that favours production of its amyloidogenic and potentially neurotoxic $A\beta$ fragment (see refs 4 and 5, for example). Moreover, transgenic overexpression of one of the mutant β APPs has resulted in the first mouse model of AD, in which age-linked cerebral deposition of $A\beta$ is accompanied by robust neuronal, astrocytic and microglial pathology⁶. In the case of chromosome 19, inheritance of the naturally occurring $\epsilon 4$ allele of the apolipoprotein E gene increases the likelihood of developing late-onset AD and lowers its age of onset⁷. Conversely, inheritance of the apolipoprotein E $\epsilon 2$ allele appears to confer a decreased risk⁷. The biochemical mechanism of these effects is unclear, but the only confirmed alteration in the AD phenotype reported in patients harbouring one or two $\epsilon 4$ alleles is a significantly increased number of $A\beta$ deposits in the brain^{8,9}.

Following the assignment to chromosome 14q of a locus that causes a particularly malignant form of familial AD with onset in the forties¹⁰, the identification of the guilty gene has been awaited with much interest and speculation. In a collaborative genetic analysis that focused in part on a group of fourteen early-onset familial AD pedigrees of diverse ethnic origin, Sherrington *et al.* used positional cloning to identify genetic regions of interest within 14q24.3 and then used direct hybridization with complementary DNA to recover transcribed sequences from these regions, including some cDNAs with complex and conserved structures that recommended them as candidate genes. By searching for sequence differences in these genes between affected members of six tightly linked pedigrees and a group of controls, the investigators ultimately came upon one gene (designated *S182*) in which five different nucleotide changes that altered the predicted amino-acid sequence occurred only in family members with AD. Strong evidence that these various missense mutations indeed represent the molecular causes of AD in their respective pedigrees comes from the findings that (1) the mutations were present in affected and some at-risk members of each family, but absent in obligate escapees and in more than 140 unrelated normal subjects; and (2) the substitutions occur within highly conserved domains of the *S182* protein and in residues that are unchanged in its murine homologue.

The primary structure of the longest open reading frame identified so far predicts a 467-amino-acid protein in both mouse and man which contains seven hydrophobic, putatively membrane-spanning domains¹. Thus *S182* appears to be an integral membrane protein having the topology of a receptor, a channel protein or a structural membrane polypeptide. The only known protein that shares substantial homology with *S182* is SPE-4, a 465-residue integral membrane protein expressed in the sperm of *Caenorhabditis elegans*. On the basis of a phenotype analysis of sterile *spe-4* mutant worms, the protein appears to be a constituent of the Golgi-derived membranous organelles of primary spermatocytes which is required for proper attachment of the organelles to the so-called fibrous bodies of these spermatid precursors¹¹. Formation of such complexes is essential for correct intracellular transport and distribution of various proteins during meiotic cell division in *C. elegans* sperm.

Assuming that *S182* may have similar properties, how could missense mutations

in this gene, several more of which are likely to be uncovered in the future, lead to an AD phenotype? Families with this genetic form of the disease develop typical AD-type neuropathology, including myriad amyloid plaques and neurofibrillary tangles¹², and they do so almost a decade earlier, on average, than families bearing mutations in β APP itself¹³. The $A\beta$ peptide, which is the principal component of the amyloid plaques in AD, is constitutively secreted by β APP-expressing cells (such as neurons, glia and endothelial cells), and cell biological studies suggest that proteolysis of β APP to release $A\beta$ occurs in mildly acidic membrane vesicles, including endosomes and late-Golgi secretory compartments¹⁴. Therefore, mutations in *S182* that alter its hypothetical role in Golgi or other membrane-trafficking intermediaries might allow β APP to stay longer in an amyloidogenic (or for less time in a non-amyloidogenic) organelle, thereby enhancing $A\beta$ production. Evidence has recently emerged^{15,16} that increased $A\beta$ secretion may indeed occur in primary cells of chromosome-14-linked donors, including some from the very families now shown to bear *S182* mutations.

Prospects

Speculating about the malfunction of a newly discovered gene whose product has not even been seen, let alone characterized, is risky. Other functions that might be disrupted by mutations in *S182* in AD might include signal transduction or ion transport¹. It could play a role that is independent of β APP and $A\beta$, but then the premature build-up of enormous quantities of $A\beta$ in these patients would require another explanation. Information about this molecule that should emerge before the year's end includes clues as to its function from structural features of both the gene (including its regulatory elements) and the protein, and hints at how it works in AD, particularly its effects on β APP metabolism.

For the burgeoning number of students

- Sherrington, R. *et al.* *Nature* **375**, 754–760 (1995).
- Goate, A. *et al.* *Nature* **349**, 704–706 (1991).
- Corder, E. H. *et al.* *Science* **261**, 921–923 (1993).
- Citron, M. *et al.* *Nature* **360**, 672–674 (1992).
- Suzuki, N. *et al.* *Science* **264**, 1336–1340 (1994).
- Games, D. *et al.* *Nature* **373**, 523–527 (1995).
- Corder, E. H. *et al.* *Nature Genet.* **7**, 180–184 (1994).
- Schmechel, D. E. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **90**, 9649–9653 (1993).
- Hyman, B. T. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **92**, 3586–3590 (1995).
- Schellenberg, G. D. *et al.* *Science* **258**, 668–671 (1992).
- L'Hernault, S. W. & Arduengo, P. M. *J. Cell Biol.* **119**, 55–68 (1992).
- Lampe, T. H. *et al.* *Ann. Neurol.* **36**, 368–378 (1994).
- Mullan, M. *et al.* *Am. J. med. Genet.* **48**, 129–130 (1993).
- Selkoe, D. J. *A. Rev. Cell Biol.* **10**, 373–403 (1994).
- Querfurth, H. W., Wijsman, E. M., St George-Hyslop, P. H. & Selkoe, D. J. *Molec. Brain Res.* **28**, 319–337 (1995).
- Scheuner, D. *et al.* *Soc. Neurosci. Abstr.* (in the press).

of AD, there is plenty left to do. Besides *SL82*, genes that cause other autosomal dominant forms of AD await identification. How each of these (as well as *ε4* of apolipoprotein E) triggers or modifies the relatively stereotyped cascade of AD pathology will need to be determined. Environmental factors that influence the course of the disease also remain to be clarified. Most importantly, small mol-

ecules that block one or another step in AD pathogenesis must be developed. Our patients, and science, will be the beneficiaries. □

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NEUROBIOLOGY

How cortex reorganizes

Jon H. Kaas

MOST of us used to be convinced that sensory areas of mature cortex in the brains of adult mammals are highly stable in their organization. This idea was consistent with evidence that visual cortex is altered by certain experiences only during a limited period of development (see ref. 1, for example). Moreover, it was presumed that processing circuits must be stable in order to perform reliably. But this assumption has been overturned by a host of experiments showing that cortical maps of receptor surfaces can reorganize after partial deactivation and after altered patterns of activation^{2,3}. Deactivation may involve damage to sensory nerves from the skin, small retinal lesions, or selective loss of hair cells of the cochlea. Regions of cortex thereby deprived of their usual source of activation typically become responsive to the remaining sen-

sory inputs. Changes in sensory use also alter cortex, but usually less dramatically. Now on page 780 of this issue, Das and Gilbert⁴ look into just how cortex reorganizes after restricted retinal lesions in cats.

Cortical reorganization could depend on the potentiation of any of several types of existing but normally ineffective neural connections, the growth of new connections, or both. Das and Gilbert⁴ propose that cortical reorganization after retinal lesion is mediated by the potentiation of a highly organized system of long-range horizontal connections in visual cortex which seem to have significant, but largely subthreshold, neuronal effects in normal animals⁵. After retinal lesions remove a competing source of activation, these subthreshold influences gradually become effective in activating neurons to suprathreshold levels. This proposal is sup-

ported by a detailed comparison of the horizontal or surface-view extent of cortex activated at suprathreshold or at suprathreshold plus subthreshold levels by a minimal visual stimulus, the restricted movement of a very small bar in the visual field.

As expected, the extent of suprathreshold activity, the spiking of neurons, measured conventionally with an array of microelectrode penetrations, was quite limited. A much larger surface area of cortex showed enhanced metabolic activity in response to the same stimulus when measured with optical imaging. As all neural events, both threshold and subthreshold, require energy, the larger region of responsive cortex measured with optical imaging includes a core region of spiking neurons and a surrounding fringe of neurons with subthreshold activity. The extent and topographic features of the fringe suggest that it depends on horizontal cortical connections. Months after binocular retinal lesions, measurements by both procedures showed that the subthreshold fringe can be driven to suprathreshold levels. Thus, horizontal connections may provide a large zone of subthreshold activity that can become suprathreshold after the deactivation of competing inputs.

Interestingly, an early and now largely abandoned procedure, that of recording evoked slow potentials with electrodes on the brain surface, may have provided comparable evidence. Surface recordings were used to reveal cortical organization during the 1940s to 1960s, but were re-

New look for old reptiles

Two historic collections of fossil reptiles return to public view this month after three-year absences. This specimen of *Triceratops* is a star turn in the revamped dinosaur galleries at the American Museum of Natural History in New York. Many familiar specimens, such as *Apatosaurus* and *Tyrannosaurus*, are displayed in new-look, dynamic poses, and the overall effect is stunning.

The renovation is part of the AMNH's continuing redesign of its fossil vertebrate galleries that started last May with 'Mammals and their Extinct Relatives', and will go on until 1996, when the hall of vertebrate origins opens. A visitor with enough time and stamina will then be able to do a circuit of the museum's fourth floor, covering the scope of vertebrate evolution. Like the mammals gallery, the dino-

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saur displays are arranged along systematic rather than chronological lines, reflecting the museum's research strengths. This is far less daunting than it sounds: technological aids such as interactive CD-ROMs help visitors pick

their way through the taxonomic jungle (and children, of course, take to both technology and technicality like *Anatitan* to water).

Another lengthy conservation effort, this time at the Natural History Museum in London, has also restored a spectacular display to public view. The NHM's unrivalled collection of Lower Jurassic marine reptiles is now back on show, the 112 mounted fossil skeletons lining the walls of a 60-metre gallery. Some of the specimens were found at Lyme Regis, Dorset, by the famous collector Mary Anning in the 1830s. Time and

heavy-handed Victorian museum techniques had taken their toll, but after extensive cleaning and restoration, the NHM's ichthyosaurs and plesiosaurs have been returned to that pristine, just-excavated state. Henry Gee

American Museum of Natural History

placed by the more precise recordings of neural spikes with penetrating microelectrodes. As with optical recording, surface electrodes reflected both spiking and non-spiking neural activity. Extracellular microelectrodes, of course, record neuronal spikes. This difference in selectivity probably accounts for many of the differences in results obtained with surface and microelectrodes. For example, recording sites in the second somatosensory area were commonly found to be activated by stimuli to either side of the body ('bilateral' receptive fields) using surface electrodes, but only from contralateral body locations with microelectrodes^{6,7}. More significantly, a small flashed spot of light, comparable in size to the small moving bar of Das and Gilbert⁴, activated a region of visual cortex, as measured with surface electrodes, that closely approximates that obtained with optical imaging⁸. So results obtained using a simple and now defunct method are in basic agreement with those derived from an elegant and technically sophisticated new approach.

However compelling we find the present results of Das and Gilbert⁴, we should remember that considerable reorganization can be expressed subcortically and that the growth of new connections may

be responsible for some components of reorganization². The formation of new connections in the mature central nervous system, previously viewed as unlikely, may in fact be very important in large-scale reactivation within sensory systems after injury and partial deprivation (for example, see refs 9 and 10). What these reactivations mean in terms of perception and performance is another matter². As brain reorganization may relate to either behavioural recovery and improvement, or to misperception and error, it is crucial to explore it further. □

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- Hubel, D. H. & Wiesel, T. N. *J. Physiol.* **206**, 419–436 (1970).
- Kaas, J. H. in *The Cognitive Neurosciences* (ed. Gazzaniga, M. S.) 51–71 (MIT Press, Cambridge, 1995).
- Kaas, J. H. *Rev. Neurosci.* **14**, 137–167 (1991).
- Das, A. & Gilbert, C. D. *Nature* **375**, 780–784 (1995).
- Gilbert, C. D. *Neurosci.* **9**, 1–13 (1992).
- Woolsey, C. N. & Fairman, D. *Surgery* **19**, 684–702 (1946).
- Nelson, R. J., Sur, M. & Kaas, J. H. *J. comp. Neurol.* **84**, 473–489 (1979).
- Woolsey, C. N. *Vis. Res. suppl.* **3**, 365–382 (1971).
- Darian-Smith, C. & Gilbert, C. D. *Nature* **368**, 737–740 (1994).
- Florence, S. L., Garraghty, P. E., Carlson, M. & Kaas, J. H. *Brain Res.* **601**, 343–348 (1993).

tracellular repeated EGF-like motifs.

In many tissues in *Drosophila*, Notch and Delta are coexpressed and they are associated with one another in vesicles⁵. They bind together in a cell aggregation assay. Furthermore, *lin-12* and *Notch* mutants function cell-autonomously, and truncated molecules devoid of the extracellular domain display constitutive activity (reviewed in refs 6 and 7). These observations suggested a receptor function for these proteins and also that the ankyrin repeats are important for transmission of the Notch signal. Interestingly, a human lymphoblastic leukaemia is associated with a translocation leading to a similarly truncated form of Notch-1. Also, Jagged-expressing cells interact with and inhibit the differentiation of *Notch-1*-expressing C2C12 cells, so that they fail to express myogenin and no longer align with one another and fuse to form myotubules³.

In *Drosophila*, precursors of the nervous system are singled out from clusters or stripes of equipotential cells expressing both *Notch* and *Delta* and the proneural genes *achaete-scute*. During this process, called lateral inhibition, physiological fluctuations in Notch–Delta signalling between neighbouring cells are amplified by a feedback loop between *Notch* and *Delta* within each cell, such that cells with increased *Delta* activity, which signal strongly, come to be surrounded by cells with raised *Notch* activity, which receive the signal (reviewed in ref. 6). Transduction of the signal results in a cessation of *achaete-scute* expression and adoption of the epidermal fate. In this way, spaced neural precursors are generated, separated by intervening epidermal cells.

It now seems that a similar process may operate in vertebrates. As in flies, *Notch* and *Delta* or *Jagged* are coexpressed in the developing nerve cord of vertebrate embryos^{1–3}. Chitnis *et al.*¹ describe the early expression of *X-Delta-1* in scattered cells of the *Xenopus* neural tube that are

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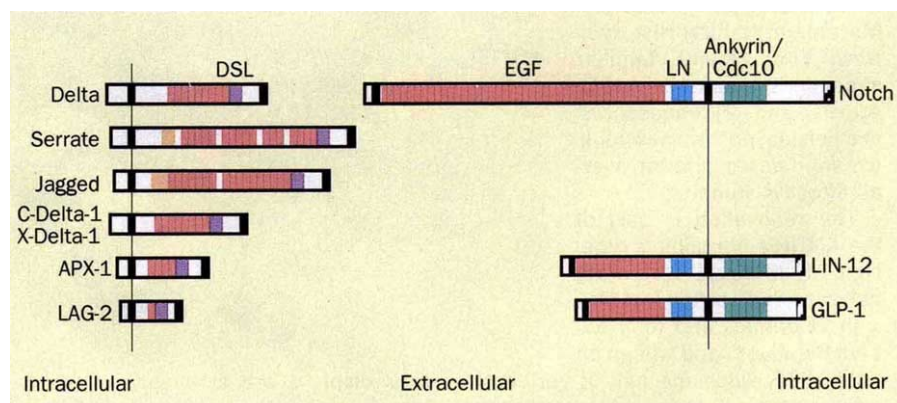
The Notch connection

Pat Simpson

EVER since its discovery in *Drosophila* by Morgan in 1916, the *Notch* gene has both puzzled and fascinated developmental geneticists. Two papers by Chitnis *et al.*¹ and Henrique *et al.*² on pages 761 and 787 of this issue and one by Lindsell *et al.* in *Cell*³ provide exciting new evidence that the *Notch* proteins have a similar function in vertebrates and invertebrates. First, the isolation of vertebrate ligands reinforces the view that Notch acts as a cellular receptor in developmental decisions. Second, it appears that, as in *Drosophila*, primary neurons of the frog nervous system arise as scattered cells in a proneural field where many cells initially have neural potential but Notch signalling restricts the proportion of cells that finally differentiate as neurons.

Notch homologues have been found in nematodes (*lin-12* and *glp-1*), frogs, fish, rodents and humans. They show a remarkable conservation of overall structure (see figure). The *Drosophila* and vertebrate proteins bear 36 epidermal growth factor(EGF)-like repeats in the extracellular domain (the *Caenorhabditis* proteins have 13 and 10 copies respectively) as well as three other cysteine-rich motifs called *Notch/lin-12* repeats. The intracellular domains display six copies of

an ankyrin/Cdc10-like motif. There are a number of invertebrate ligands: Delta and Serrate in *Drosophila* and LAG-2 and APX-1 in *Caenorhabditis*. The newly described vertebrate ligands, Delta in the chick² and the frog¹ and Jagged in the rat³, are very similar to the invertebrate proteins (see figure). They all have in common a DSL motif⁴ critical for Notch binding and a variable number of ex-



The structure of the receptors Notch, LIN-12 and GLP-1 and of their ligands Delta, Serrate, Jagged, C-Delta-1, X-Delta-1, APX-1, and LAG-2. The conserved motifs (EGF-like repeats, ankyrin/Cdc10-like repeats, Notch/LIN-12 (LN) repeats) and the DSL (for *Delta*, *Serrate* and *lag-2*; ref. 4) region are highlighted in different colours. The intracellular domains of the ligands display no significant homology. PEST sequence is in single-letter amino-acid notation.

destined to become the primary neurons. *X-Delta-1* expression precedes that of early neuronal markers such as *N-tubulin* and disappears as the neurons differentiate. It thus coincides with commitment of cells to the neural fate. Overexpression of *X-Delta-1* inhibits the production of primary neurons. This also occurs with expression of a truncated, activated form of *X-Notch-1*, indicating that *X-Delta-1* suppresses neurogenesis by activating *Notch*. In contrast, an antimorphic form of *X-Delta-1* causes the opposite phenotype, in which neurons are extensively overproduced: these are exactly the same phenotypes as those seen in *Drosophila* with loss-of-function *Notch* and *Delta* mutants or gain-of-function *Notch* mutants (reviewed in ref. 7).

The authors also note that activated *X-Notch-1* inhibits the expression of *X-Delta-1*, creating a potential feedback loop that would reinforce differences between adjacent cells. Furthermore, the neural hyperplasia in *Xenopus* is restricted to precise domains, perhaps similar to the proneural domains of *Drosophila*. Indeed, the vertebrate *achaete-scute* genes *XASH-3* and *MASH-1* are expressed here.

Notch-mediated lateral signalling is not restricted to neural tissues. The singling out of one cell from an equivalence group seems to be a process common to a number of tissues in *Drosophila* such as the segregation of muscle founder cells and of the tip cells of malpighian tubules. Similarly, in *Caenorhabditis*, *lin-12* controls a number of cell-fate decisions between equivalent cells⁶. *Notch* and *Delta* are widely distributed in vertebrate embryos. Recently a model implicating *Notch* in a process of lateral inhibition in the generation of somite precursors in the mouse has been proposed⁸.

Many questions remain, but it is apparent that the *Notch* proteins define a new type of cellular receptor whose function is specifically implicated in the process of lateral signalling. This is another example of the remarkable conservation throughout evolution of basic mechanisms of development. □

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Melt pathways in the mantle

Marc Hirschmann

OCEANIC ridges are the dominant sites of energy and mass transfer from the Earth's interior to the crust, hydrosphere and atmosphere, but many key processes beneath ridges remain poorly understood. One outstanding problem is how partial melts in the asthenosphere concentrate into channels — initially, melts must leave their source by intergranular percolation, but there is considerable evidence that they are rapidly channelled into larger pathways. What mechanism focuses melt flow, and what is the geometry of melt channels? And how effectively does flow of melt in the asthenosphere mix partial melts formed at different depths?

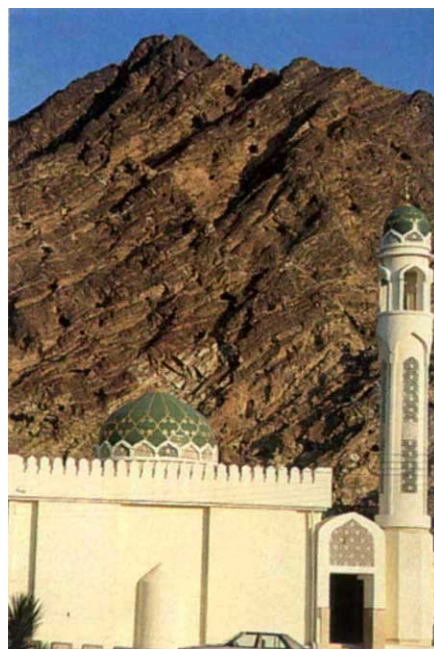
On page 747 of this issue¹, Kelemen *et al.* present geochemical and structural observations from the Oman ophiolite that go some way towards answering these

questions. The new results suggest that focused melt pathways in the asthenosphere are marked by bodies of dunite; that the dunite pathways are formed, at least in part, by chemical reaction between ascending melts and peridotite wall-rock; and that extensive mixing takes place during ascent in these channels. The results will provide petrologists and geophysicists with new avenues for investigating melt transport in the mantle.

Because of its large size and its excellent preservation, the Oman ophiolite (see photograph) is one of the best natural laboratories for understanding mantle processes. As in most ophiolites and other mantle samples, the mantle section at Oman contains numerous dunite pods. Kelemen *et al.* use electron and ion microprobe analyses of mineral grains to show that the dunite pods in Oman equilibrated with liquid similar to mid-ocean-ridge basalt (MORB), but that peridotite surrounding the pods did not; structural relationships indicate that the dunite pods originated in the asthenosphere. The combined chemical and structural evidence therefore suggests that MORB-like liquids reacted with the dunite in the asthenosphere. Equilibration of well-mixed MORB with dunite pods, but not with the surrounding wall-rock, is best explained if the dunite bodies are channels for melt transport.

The link between melt pathways in the asthenosphere and identifiable geological features provides the opportunity to test proposed mechanisms of melt transport against petrological observations. Kelemen *et al.* favour the idea that channels form by chemical reaction between ascending melts and peridotite, resulting in preferential dissolution of pyroxene, increased porosity and enhanced reaction. Small perturbations of porosity therefore lead to increased flow and to formation of channels composed of dunite². Alternatively, mantle dunite could be the by-product of other melt transport mechanisms. Regions of high porosity may be generated by solitary waves³ or by shearing of viscously deforming partially melted peridotite⁴, and the large volumes of fluid that flow through such regions would also preferentially dissolve pyroxene and leave behind dunite¹, thereby increasing porosity and flow. Sorting out which mechanism (or combination of mechanisms) is responsible for melt transport and dunite formation in the asthenosphere will require fluid dynamical models that incorporate chemical reaction between matrix and melt, as well as more detailed investigation of mantle dunite.

Whatever the specific origin of these



Towering above this mosque is part of the Oman ophiolite, the geological body that is the subject of the paper by Kelemen *et al.*¹ discussed here. Ophiolites are fragments of oceanic lithosphere that have been thrust onto land during tectonic collisions, and one of the largest and best preserved is extensively exposed along the northeast coast of the Arabian peninsula in the sultanate of Oman. The Oman ophiolite is thought to have formed at a mid-ocean ridge, and the new observations of Kelemen *et al.* suggest that bodies of dunite in the mantle section, here seen as light coloured bands interlayered with darker residual peridotite, are important pathways for melt transport in the mantle beneath mid-ocean ridges. The photograph, taken near Muscat, the capital of Oman, shows part of the mantle section that has a strong foliation and corresponds to the bottom of the schematic cross-section shown in Fig. 1 of the paper (page 748).

1. Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D. & Kintner, C. *Nature* **375**, 761–766 (1995).
2. Henrique, D. *et al.* *Nature* **375**, 787–790 (1995).
3. Lindell, C. E., Shawber, C. J., Boulter, J. & Weinmaster, G. *Cell* **80**, 909–917 (1995).
4. Tax, F. T., Yeager, J. J. & Thomas, J. H. *Nature* **368**, 150–154 (1994).
5. Kooh, P. J., Fehon, R. G. & Muskhavitch, M. A. T. *Development* **117**, 493–507 (1993).
6. Greenwald, I. & Rubin, G. M. *Cell* **68**, 271–281 (1992).
7. Artavanis-Tsakonas, S., Matsuno, K. & Fortini, M. E. *Science* **268**, 225–232 (1995).
8. Conlon, R. A., Reaume, A. G. & Rossant, J. *Development* **121**, 1533–1545 (1995).

pathways, future field studies of mantle dunite may provide key constraints on the distribution and dimensions of asthenospheric melt channels. Understanding the role and origin of mantle dunite will also require determination of the integrated flux of melt through these channels. Kelemen *et al.* estimate that the volume of melt percolating through the dunite pods may exceed the dunite volume by a factor of 10, and that locally it may reach a factor of 300–400, but additional field and theoretical studies are needed to generate robust constraints. Metamorphic petrologists have developed techniques for calculating integrated water/rock ratios from petrological observations, and analogous geochemical tests for determining integrated basalt/dunite ratios should be sought. Also, it is not yet established whether porous dunite channels can deliver melt from the site of melting to the surface on the thousand-year timescale required by observed ^{226}Ra – ^{230}Th – ^{238}U disequilibria in MORB (ref. 5).

Finally, evidence that dunite channels

equilibrate with MORB-like basalt sheds light on the locus of MORB aggregation and mixing, but also raises questions about the role of dunite bodies as melt pathways. Melting in the sub-ridge mantle is a fractional process that should generate an exceptionally wide range of liquid compositions⁶. As MORBs do not reflect much of this compositional variability, they must be products of extensive mixing processes. It is commonly assumed that much of this blending takes place in lithospheric magma chambers, but there are few constraints on magma mixing in the asthenosphere.

The mineral chemistry of the Oman dunite pods indicates that extensive mixing takes place during transport in the asthenosphere and that MORB gains much of its geochemical character before it enters the lithosphere. On the other hand, melt inclusions in olivine phenocrysts from MORB commonly preserve both ultra-depleted and ultra-enriched liquids that have experienced little or no mixing^{7,8}. The unmixed character of these

inclusions suggests that some melts are delivered from disparate parts of the melting regime to the lithosphere by processes different from those recorded in the Oman dunite pods. The evidence from Oman shows that dunite channels are significant melt pathways in the asthenosphere. But they may not be the only ones. □

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1. Kelemen, P. B., Shimizu, N. & Salters, V. J. M. *Nature* **375**, 747–753 (1995).
2. Kelemen, P. B., Whitehead, J. A., Aharanov, E. & Jordahl, K. A. *J. geophys. Res.* **100**, 475–496 (1995).
3. Barclon, V. & Lovera, O. J. *Fluid Mech.* **204**, 121–133 (1989).
4. Stevenson, D. J. *Geophys. Res. Lett.* **16**, 1067–1070 (1989).
5. Rubin, K. H. & MacDougall, J. D. *Nature* **335**, 158–161 (1989).
6. Johnson, K. T. M., Dick, H. J. B. & Shimizu, N. *J. geophys. Res.* **95**, 2661–2678 (1990).
7. Shimizu, N. *Min. Mag.* **58A**, 829–830 (1994).
8. Sobolev, A. V. & Shimizu, N. *Nature* **363**, 151 (1993).

OBITUARY

Raymond Lyttleton (1911–95)

THE death on 16 May of Raymond Arthur Lyttleton opens a large gap in the collective knowledge of the British astronomical community. For Ray Lyttleton, essentially alone, held at his fingertips the celestial mechanics of Newton's successors in the nineteenth century. In particular, his knowledge of the theory of the Moon's motion, the only problem which Newton said had ever made his head ache, was without compare among his colleagues. In his career, spent from 1969 as professor of theoretical astronomy at the University of Cambridge, Lyttleton ranged widely, tackling issues in both cosmology and geophysics.

It was never clear, even to those who knew him well, whether his research interests were decided by a sense of style or whether in his early years it was the research that developed the style. Most scientists tend to stumble towards a problem, gradually clarifying their perceptions of it as time goes on. Lyttleton, on the other hand, chose investigations where he could begin with issues that were clearly and explicitly defined, putting himself in the position of a student who is called upon to solve an especially taxing problem in some celestial mathematical examination.

But the exam was invariably without a time limit. For a problem that was hard enough and interesting enough, he was prepared to go on trying for a solution over decades of effort. It was so with the question of the rotational instability of an incompressible fluid, which had caused difficulty for mathematicians as diverse

as Jacobi, Poincaré and Jeans. The culmination of this work was published as *The Stability of Rotating Liquid Masses* in 1953.

Lyttleton's interests in astrophysics

were mainly concerned with situations in which he considered that dynamics had an important role to play, as in the relationship between the gravitational field of a star and the interstellar gas through which it moves. Such an interplay causes the stars to be open systems, a view that seemed strange when it was first advanced at the beginning of the 1940s. Here again the accretion problem, as it has become called, continued to be investigated in its fundamentals for two decades or more after the Second World War. And in its practical details it

has continued to influence astrophysics.

Lyttleton's contribution to the understanding of the highly complex practical dynamics problem of the flight of a cricket ball has become well known. Almost alone among mathematicians with an interest in ball games, he was a considerable practical performer in his own right (though finding it difficult to devote as much time to sport as perhaps he would have liked).

There were few who knew Lyttleton who did not at some stage encounter the razor edge of his wit. He delighted in pointing out self-contradictions in the rules governing organizations with a high measure of formal respectability — clubs, learned societies, colleges and the like. Yet, paradoxically enough, he was thoroughly formal himself on formal occasions. And so far from being a dangerous customer, as many took him to be, when called on to occupy various posts he discharged them with a minimum of difficulty and fuss — as when for a number of years he was the Secretary of the Faculty of Mathematics at Cambridge, and Geophysical Secretary of the Royal Astronomical Society.

On almost all matters, whether in science or out, Ray Lyttleton had his own way of looking at things. One can say without fear of error that he never sought to enhance his reputation by believing what he was told. Fred Hoyle

Sir Fred Hoyle was formerly the Director of the Institute of Theoretical Astronomy, Cambridge, UK.

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A fine set of whiskers

Philip Ball

THE ancient practice of putting chopped horsehair into plaster is a venerable example of how 'whisker reinforcement' toughens brittle materials. The engineering principle is older still: the paradoxical toughening of composite materials by the incorporation of weak interfaces presumably gave molluscs the same protection from predators millions of years ago as it does today.

Ceramic materials reinforced with fine whiskers of strong materials such as silicon carbide are typically twice as tough as the pure matrix material. Generally these whiskers have diameters of about half a micrometre, but on page 769 Charles Lieber and colleagues show how to make carbide whiskers ten to a hundred times thinner. As the toughness of a whisker-reinforced composite tends to increase with increasing whisker content, these ultrafine whiskers could provide the binder for still tougher composite materials.

The origin of whisker toughening lies in the strategy that it is better to yield a little than to risk catastrophic collapse. High strength in a material, as reflected in the bond energies that hold together the atomic lattice, is not enough by itself to prevent failure through cracking, because the stresses that accumulate at the tip of a propagating crack can be phenomenal. Rather than trying to prevent the progress of a crack tip, it is therefore better to absorb its energy by incorporating into the material weak interfaces that will fail when a crack reaches them. The crack then expends its energy in bringing about localized, small-scale failure rather than brittle fracture. In whisker composites, this dissipation of energy is primarily the

consequence of whisker pull-out as the two faces of the crack are pulled apart to expose ever more of the whiskers bridging them.

The fine whiskers made by Lieber and colleagues might be regarded as fossilized carbon nanotubes: they 'mineralize' the tubes through a reaction between the graphitic shells and volatile oxides or halides of the metallic component. The gases react to form a carbide structure that retains the general dimensions of the tubes; but the reaction proceeds to fill up the hollow tube interiors, turning them into solid rods. These structures are highly crystalline, with the result that they exhibit faceted surfaces. When the fastest-growing crystal facets do not lie more or less parallel to the tube axis, the result is a sawtooth structure, as shown in the photo-

graph, the crimped surface of which would be expected to have a very high pull-out energy in composite materials. Mineralization of helical carbon nanotubes produces helical rods; it cannot be long before someone measures the mechanical properties of these strong 'nanosprings'.

The rods possess the same properties as the parent bulk phase: the iron carbide rods are ferromagnetic, the niobium carbide rods are superconducting, the titanium carbides are metallic. This raises the possibility of engineering intriguing properties into composite materials, for example by varying the nanorod content around the percolation threshold at which a network of metallic fibres forms a continuous conducting pathway through the sample. Such ideas have already been explored in carbon-fibre technology for making smart materials that show a large conductivity response to the appearance of small flaws. □

Philip Ball is an associate editor at Nature.

MULTIPLE SCLEROSIS

Presenting an odd autoantigen

Lawrence Steinman

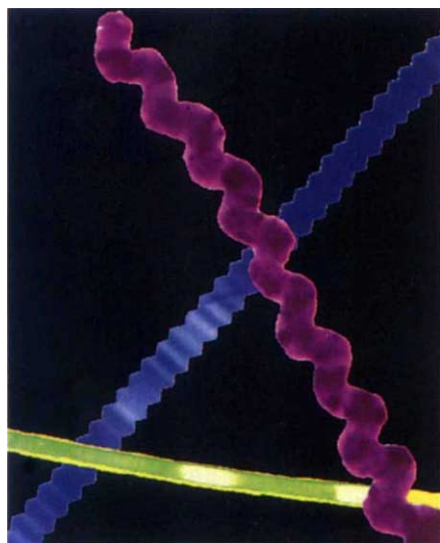
MULTIPLE sclerosis (MS) is an autoimmune disease involving an integrated attack by T cells, B cells and macrophages on the myelin sheath that surrounds nerve fibres. Potentially inflammatory cytokines such as γ -interferon and tumour-necrosis factor- α are found at the sites of damage, and T cells and antibodies directed against myelin components can be isolated from inflamed regions in the central nervous system. Attempts to identify the components of the myelin sheath that provoke this misguided arsenal have yielded a number of candidates. On page 798 of this issue¹, van Noort *et al.* describe the isolation of a protein that is a prominent target at the disease site in the myelin sheath: this autoantigen turns out to be a small heat-shock protein, α B-crystallin, which is induced in the diseased white matter.

van Noort and colleagues reasoned that there could be an antigen present in the white matter of MS brain that is not found in normal white matter and which specifically activates T cells. They separated the proteins of the myelin sheath using reversed-phase high-performance liquid chromatography and discovered that a particular fraction in the myelin of MS brain, but not in the myelin taken from healthy brain, stimulated proliferation of T cells, causing them to release the pro-inflammatory cytokines γ -interferon and interleukin-2. α B-crystallin was identified as the myelin protein that elicited the strongest immune response. They then showed that α B-crystallin is expressed

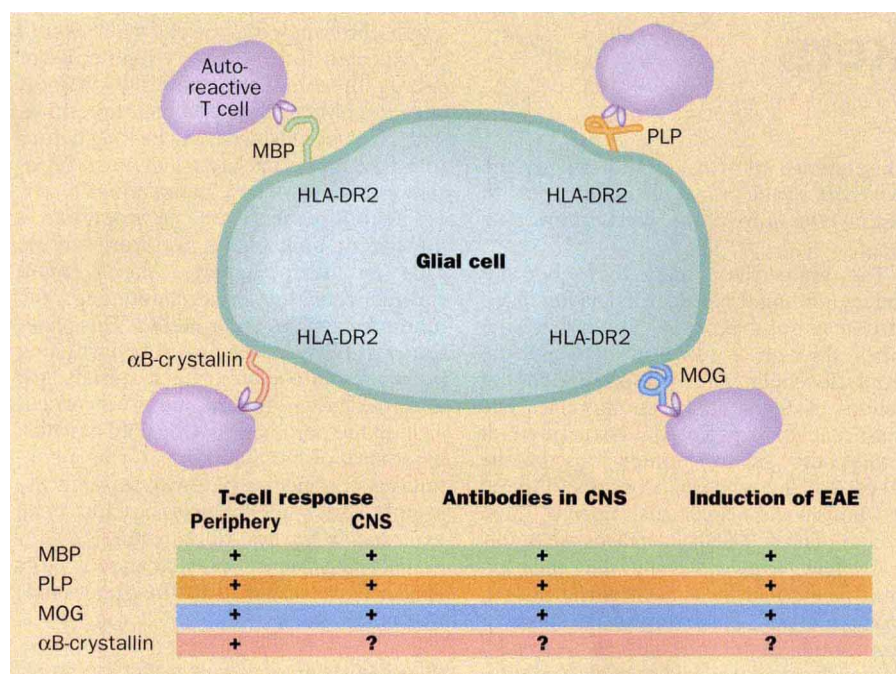
in glial cells from MS lesions but not in white matter from healthy individuals or in unaffected white matter from MS brain. α B-crystallin was found in oligodendroglial cells as well as in astrocytes in plaques from patients with acute and chronic MS.

These exciting results provide a strong case for α B-crystallin as a target of the immune response in MS, but they raise the question of the precise role of α B-crystallin in the pathogenesis of the disease. α B-crystallin is constitutively expressed in the lens of the eye, myocardial cells and kidney epithelium, and is inducible not only in MS tissue, but also in brains of patients with other neurological diseases, including Alzheimer's, Parkinson's and Huntington's diseases¹. Degenerative diseases like Huntington's and Parkinson's do not appear to be mediated by the immune system, so it is not clear why α B-crystallin should be immunogenic in MS but not in these other degenerative conditions. One possibility is that the induced expression of α B-crystallin may be a reaction to some neuropathological stimulus that is not unique to MS. In MS additional local events may be required to allow the development of an immune response to α B-crystallin.

To assess the significance of the immune reaction against α B-crystallin in the pathology of MS, the immune response to other myelin components in MS needs to be considered. Myelin basic protein, proteolipid protein, transaldolase and 2',3'-



Three varieties of nanorod as prepared by Lieber and colleagues, each just a few nanometres in width.



Recruitment of autoreactive T cells by different components of the myelin sheath (dark green) in white matter from MS brain (MBP, myelin basic protein; PLP, proteolipid protein; MOG, myelin oligodendroglial glycoprotein), and the immune responses triggered by these components in MS patients and in laboratory animals. EAE, experimental allergic encephalomyelitis.

cyclic nucleotide 3'-phosphodiesterases, as well as two members of the immunoglobulin supergene family found in the myelin sheath (myelin oligodendroglial glycoprotein and myelin-associated glycoprotein) all trigger immune responses in MS patients². When they are injected into laboratory animals, these myelin components cause allergic encephalomyelitis, with inflammation and demyelination in the central nervous system (see figure). In addition, other inducible heat-shock proteins can be detected in glial cells in MS lesions and can stimulate an immune response in MS patients³⁻⁵. The large number of antigens capable of eliciting an immune response in MS patients may represent the intermolecular dispersion of an immune response that arose initially against a single component of myelin. Immune reaction to different components of a supramolecular structure, such as the myelin sheath in MS or the mitochondrion in primary biliary cirrhosis, is common in individuals with autoimmune disease that involves a discrete organ.

We cannot yet say which of these antigens is most important in the pathogenesis of MS. Strong evidence is emerging that an immune response to certain regions of myelin basic protein and proteolipid protein could be critical in MS (see figure). The major T- and B-cell response in the central nervous system of MS patients who carry the human leukocyte antigen HLA-DR2 (about two-thirds of patients) is directed to a region between residues 84 and 103 of myelin basic protein^{6,7}. The principal antibody response in the lesions of MS patients is also directed against this

region⁸ and microbes that share critical sequence homologies with it can provoke a response to this myelin peptide⁹. Increased numbers of T cells reactive to proteolipid protein are also found in the spinal fluid of patients with MS⁷. The response to αB-crystallin has not yet been checked for T and B cells in the brain.

These efforts to determine which antigens trigger the pathological response in MS brain have very practical consequences. It is now possible to induce immunological tolerance to specific proteins using a variety of strategies, including the alteration of peptide ligands that bind to the T-cell receptor and the blockade of costimulatory molecules on T cells^{2,10}. All of these approaches have been effective in suppressing animal models of MS. Identification of the critical proteins involved in MS permits the testing of these strategies for the induction of tolerance in patients suffering from this perplexing disease. □

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1. van Noort, J. M. *et al.* *Nature* **375**, 798–801 (1995).
2. Steinman, L., Waisman, A. & Altmann, D. *Molec. Med. Today* **1**, 79–83 (1995).
3. Selmaj, K., Brosnan, C. & Raine, C. S. *Proc. natn. Acad. Sci. U.S.A.* **88**, 6452–6456 (1991).
4. Wucherpfennig, K. W. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **89**, 4588–4592 (1992).
5. Hvas, J. *et al.* *J. Neuroimmun.* **46**, 225–234 (1993).
6. Oksenberg, J. *et al.* *Nature* **362**, 68–70 (1993).
7. Zhang, J. *et al.* *J. exp. Med.* **179**, 973–984 (1994).
8. Warren, K., Catz, I., Johnson, E. & Mielke, B. *Ann. Neurol.* **35**, 280–289 (1994).
9. Wucherpfennig, K. & Strominger, J. *Cell* **80**, 695–705 (1995).
10. Weiner, H. W. *et al.* *A. Rev. Immun.* **12**, 809–837 (1994).

DAEDALUS

Plain polymer

LAST week Daedalus was studying the reaction of graphite to the linear carbon polymer, carbyne. He is now musing on its reaction with fluorine. This gives the saturated polymer, graphite fluoride — infinite sheets of carbon hexagons, with a fluorine on each carbon atom.

In principle, hydrogen should react with graphite in the same way. The result would be a novel hydrocarbon sheet polymer, a sort of two-dimensional polyethylene. This doesn't happen; even at high temperatures hydrogen merely etches graphite away into hydrocarbon vapours. Daedalus reckons that the saturation of graphite must go via negative ions. Fluorine is a ready source of the F^- ion, whereas hydrogen is a very poor source of H^- . To make the reaction go, extra electrons must somehow be pumped into the graphite.

Chemically, this might perhaps be done by electrolysis of molten lithium hydride with a graphite anode. But Daedalus has a bolder scheme. Graphite is a conductor; raise it to a high enough negative voltage and its electron density should increase to the point where it reacts with hydrogen.

Charge concentrates at the surface of a conductor, and its density is greatest on a sharply curved surface. So Daedalus is taking very fine carbon fibres, and raising them to enormous negative voltages in an atmosphere of hydrogen, pressurized to inhibit sparking or corona discharge. Graphite hydride should form on the fibres.

The new polymer may adhere as a strong coating, gradually thickening as the reaction proceeds inwards. If so, a carbon fibre continuously moving through the reaction chamber will be converted into one of graphite hydride. But the charged product may instead be blown off the fibre by strong electrostatic repulsion as fast as it forms; in this case it will accumulate as a powder.

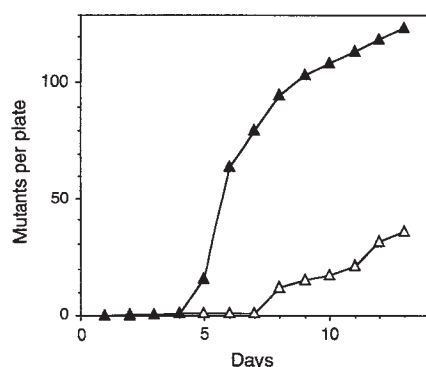
Like graphite itself, graphite hydride should slowly anneal at high temperature and pressure to a well formed crystalline product. It will be a sort of organic mica: infusible, lamellar, unreactive, very tough in the plane of the molecular sheets and very impermeable at right angles to it. Daedalus expects its major impact to be on food packaging. The tin can, that ancient invention, survives only because no present-day plastic film is quite impermeable to air. But graphite hydride, with its monolithic sheet molecules, will be a perfect gas barrier. Soup, beans, sardines, beer, all will be sold in flexible pouches of thin-film graphite hydride, while cans and can-openers are relegated to museums of technology.

David Jones

mutY 'directs' mutation?

SIR—The ability of cells to undergo spontaneous mutation while in the resting state was demonstrated by Ryan *et al.*¹ with *Escherichia coli* more than 30 years ago, but received little attention until the report by Cairns and collaborators² that the mutations that arose were 'directed', that is, they specifically overcame whatever starvation was imposed to maintain them in a non-growing state.

Apparently, 'directed' mutation to prototrophy is readily demonstrated in certain ochre auxotrophs starved of their require-



Appearance of tyrosine-independent mutants of bacteria incubated on plates lacking a required amino acid: influence of *mutY*. Bacteria were incubated overnight on oxid nutrient broth no. 2, centrifuged, resuspended in buffer and plated on minimal agar plates supplemented with 0.4% glucose and 10 $\mu\text{g ml}^{-1}$ leucine, WU3610 *tyrA14* (Δ) and its *mutY68::kan* derivative CM1321 (▲) (initial inoculum 10^8 bacteria per plate) (means of at least 3 experiments).

ments. The prototrophs that arise are slow-growing suppressors inferred to have arisen by transversion at G:C base pairs and different from those commonly found to arise in growing bacteria³. This specificity, coupled with the time-dependent manner in which mutations arise on starvation plates, led to the suggestion that they are due to a DNA lesion. The lack of effect of DNA repair mutations such as *recA*, *umuC*, *lexA* and *uvrA* implied that this hypothetical lesion is a non-bulky type of damage generating mutations by miscoding rather than error-prone repair³.

A candidate for the hypothetical lesion in DNA is 7,8-dihydro-8-oxoguanine (8-oxo-G), as this is known to cause G:C to T:A transversions by incorporation of adenine opposite it, and is found in detectable quantities in cellular DNA. To mitigate its effect, bacteria have developed several enzymes including MutY, a glycosylase that removes adenine residues erroneously incorporated in DNA opposite 8-oxo-G.

Introduction of *mutY* deficiency into the *tyrA14* ochre strain WU3610 resulted in a dramatic increase in starvation-associated mutants (see figure). The excess mutants

were slow-growing suppressors similar to those observed in the *mutY*⁺ strain, showing that they can, as predicted, be caused by 8-oxo-G. If the suppressor mutants that arise in the *tyrA14* ochre strain under starvation conditions are at the anticodon of a transfer RNA locus, then the 8-oxo-G can be formed on the transcribed strand. Miscoding during transcription would result in a transient Tyr⁺ phenotype, which could allow a round of DNA replication to occur. This round could permanently fix the mutation by a DNA mispairing event.

Such a mechanism could explain why starvation-associated mutation gives the appearance of being 'directed' in these strains. DNA replication is needed to fix the mutation by a mispairing event opposite 8-oxo-G, but is inhibited by the restriction of protein synthesis due to the amino-acid auxotrophy. Only genes where mispairing opposite 8-oxo-G during transcription can give rise to a transiently prototrophic phenotype will ever have the opportunity to mispair during DNA replication.

It is not impossible that an analogous situation could exist in nondividing somatic cells of higher organisms. One might envisage a proto-oncogene that could trigger a cell replication cycle if a mispair occurred opposite an 8-oxo-G residue and if the 8-oxo-G were in the transcribed strand. In the *TP53* gene, for example, among more than 350 independent point mutations found in a wide variety of human cancers, there were 11 transversions where a guanine residue was in the transcribed strand⁴.

The hypothesis of transcriptional miscoding by 8-oxo-G provides a plausible explanation of the extent of starvation-associated mutation in *mut*⁺ cells and its apparently 'directed' nature. Although this hypothesis is not expected to explain all observations of 'directed' mutation, transversions at G:C sites where the G is in the transcribed strand were by far the most common base-pair substitutions in a study of starvation-associated mutation with the set of *lacZ* strains of Cupples and Miller⁵. A similar phenomenon could in principle occur with other miscoding lesions in mutation systems that could respond to lesions on the transcribed strand. The data of Mackay *et al.*⁶ implicating *O*⁶-alkylguanine in the induction of G:C to A:T transitions in stationary phase cells are entirely consistent with this interpretation.

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1. Ryan, F. J. *et al.* *J. gen. Microbiol.* **30**, 193–199 (1963).
2. Cairns, J. *et al.* *Nature* **335**, 142–145 (1988).
3. Bridges, B. A. *Mutat. Res.* **307**, 149–156 (1994).
4. Caron de Fromental, C. & Soussi, T. *Genes Chrom. Cancer* **4**, 1–15 (1992).
5. Hall, B. G. *Genetica* **84**, 73–76 (1991).
6. Mackay, W. J. *et al.* *J. Bact.* **176**, 3224–3230 (1994).

Life at extremely low pH

SIR—Only four organisms, oddly enough all eukaryotes, have so far been reported to grow at pH values around zero: the thermoacidophilic coccoid rhodophyte *Cyanidium caldarium*¹; the fungi *Acontium cylatium*; *Cephalosporium* sp.²; and *Trichosporon cerebriae*³. We have now identified two related species of archaea from Japanese solfataras which can grow at and slightly below pH 0.

The most acidophilic prokaryote previously described is the archaeal mycoplasma *Thermoplasma acidophilum*, which barely grows at pH 0.4 and optimally thrives between pH 1.8 and 2 (ref. 4). We have found two species of a novel genus of coccoid, hyperacidophilic, moderately thermophilic archaea, *Picrophilus oshimae* and *Picrophilus torridus* (DSM numbers 9789 and 9790, respectively), in samples from moderately hot (about 60 °C) but extremely acidic places, a situation frequently encountered in solfataric fields. The organisms were isolated as single colonies on starch plates, both clearly growing around pH 0 (the lowest pH was -0.06 ± 0.01 measured at 20 °C) and optimally at pH 0.7 (Fig. 1), the lowest pH optimum known to date. The optimal growth temperature was 60 °C. Both organisms were aerobic, nonfermenting heterotrophs, utilizing yeast extract and unable to grow by sulphur oxidation and reduction, or by sulphur respiration.

Phylogenetically, the closest relative of *Picrophilus* is *T. acidophilum*. The 16S ribosomal RNA of *P. oshimae* (GenBank/EMBL accession number 84901) shows 90.7% sequence identity with that

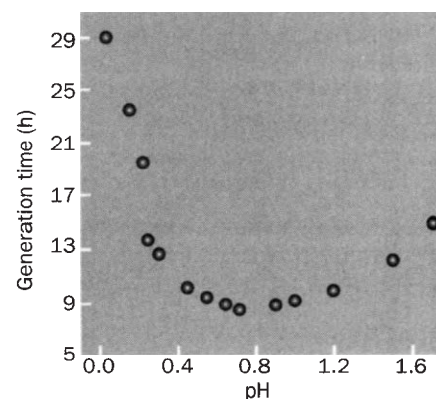


FIG. 1 Optimal pH of *P. oshimae*, isolate KAW2/2. Doubling times were calculated from the slopes of growth curves (not shown). The medium (according to E. A. Freundt⁷) contained 0.2% yeast extract as carbon source. 1 ml each of late-logarithmic cultures grown under optimal conditions (60 °C and pH 0.7, aeration by moderate shaking) was used to inoculate 50 ml of preheated growth media of varying pH, measured with an Ingold Mettler electrode (type 405-60-S7/120 for pH 0 to 12). 1 M HCl was used as a reference for pH 0.

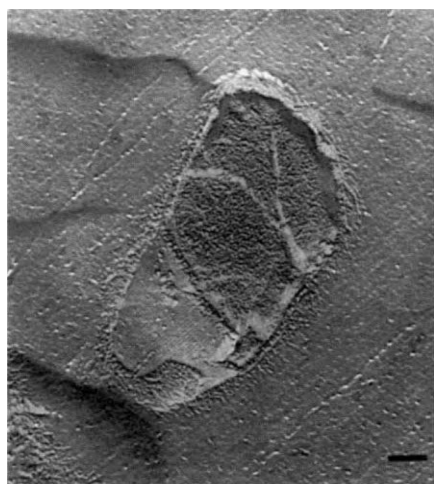


FIG. 2 Freeze-etched cells of *P. oshimae* showing the tetragonal S-layer. The more pronounced relief is due to prolonged (deeper) etching of the specimen. Bar, 200 nm. The concentrated cell suspension was frozen between two copper platelets in a cryojet (Balttec Jet Freeze Device FDJ030). Freeze-etching was carried out in a Balzers freeze-etch unit (BAF 400, Balzer AG) at -100°C for 30 s. The fracture faces were shadowed with 2 nm of platinum-carbon at an elevation angle of 45° . The metal film was backed with a 10-nm carbon layer. Replicas were washed with water and kept overnight in 70% H_2SO_4 .

of *T. acidophilum* and about 97% identity with a partial sequence of *P. torridus*. In contrast to *T. acidophilum*, both isolates possess a filigree tetragonal S-layer (Fig. 2) and a brush-like outer layer of short filaments possibly representing polysaccharide chains (H. Engelhardt, personal communication).

The G+C content of these isolates was found to be 36% as opposed to 46% for *T. acidophilum* and 38–40% for *T. volcanium*⁵. The DNA-dependent RNA polymerase of *P. oshimae* did not crossreact with an antibody against that of *T. acidophilum* in the Ouchterlony immunodiffusion assay⁶. These differences suggest that *Thermoplasma* and *Picrophilus* represent different families of the order Thermoplasmatales. The bisphityl tetraether lipids are dominated by a single phosphoglyco component and resemble those of *T. acidophilum* (A. Gambacorta, personal communication).

One important physiological question

raised by these findings is how these hyperacidophiles cope with their harsh environment—with a strong proton pump or a low proton membrane permeability?

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Star clusters in merging galaxies

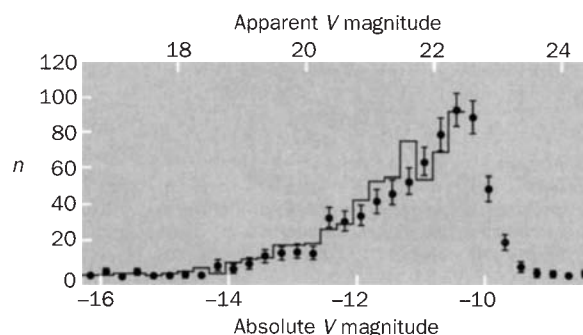
SIR — Whitmore and Schweizer¹ showed that the merging galaxy pair NGC4038 and NGC4039 contains hundreds of young, luminous, 'super' star clusters (SSCs) and hypothesized that these may be proto-globular clusters. van den Bergh² challenged this interpretation by arguing that the distributions of luminosities and sizes of the SSCs are unlike those expected for young globular clusters. I will show that this hypothesis is not yet refuted.

The Hubble Space Telescope (HST) is discovering ever more SSCs. Their hosts can be classified as starburst galaxies, many of which, like NGC4038/4039, are merging systems. When the nearest star bursts are examined, it is clear that SSCs are themselves highly clustered. For example, there are about 50 SSCs in region A of the prototypical starburst M82 (ref. 3). Their nearest-neighbour projected separation is typically ≤ 18 pc (assuming a distance $D = 3.6$ Mpc). Whitmore and Schweizer estimated cluster half-light radii, R_{eff} , from HST images by modelling the difference in brightness of the cluster in two circular apertures of projected radii 7 and 43 pc for $D = 29$ Mpc. Their large-aperture measurements are thus likely to be contaminated by neighbouring SSCs, which would inflate their R_{eff} estimates. The SSCs in M82 typically have $R_{\text{eff}} = 1.8$ pc (ref. 3) if one assumes their intrinsic profiles to be gaussian. Thus, when one considers the nearest SSCs, the measured R_{eff} sizes are consistent with globular clusters that typically have $1 \leq R_{\text{eff}}/(1 \text{ pc}) \leq 6$ (ref. 2).

Galactic globular clusters are so old compared with their age spread that they are of effectively the same age, and the globular cluster luminosity function reflects only the mass function. The comparison shown by van den Bergh assumed that the SSCs are also of the same age.

The blue colours and associated emission-line nebulae of some of the SSCs, however, indicate that cluster formation is still occurring in NGC4038/4039 and probably has been since the two galaxies made their last close approach about 200 million years ago⁴. An SSC's V-band luminosity is expected to fade by 1.7 mag in its first 200 Myr (ref. 5). This will significantly broaden the luminosity function and push its peak to a fainter magnitude than the luminosity function shown by van den Bergh.

Comparison of the NGC4038/4039 SSC luminosity function with a Monte-Carlo simulation of forming globular clusters provides a good representation of the observed luminosity function (see figure). Simulations with the same assumptions also match the SSC colour-magnitude and two-colour diagrams of Whitmore and Schweizer. The hypothesis that the clusters in NGC4038/4039 are young globular clusters can thus account for the observed cluster luminosity function. Also, crowding effects are likely to have inflated Whitmore



The V-band luminosity function of super star clusters in NGC4038/4039. The number (n) of clusters at a given magnitude is counted in 0.5-mag bins. The top axis shows apparent magnitude, the bottom axis absolute magnitude. A uniform foreground extinction of $A_V = 0.5$ mag is assumed⁴. The data points are the observations taken from Table 1 of ref. 1, with error bars showing $\sqrt{n}$. The solid line shows the simulation, including only the clusters with $m_V > 22.5$. The SSCs are assumed to have formed at a constant rate for 200 Myr, with a globular cluster-like initial mass function taken to be a gaussian in logarithmic mass, centred at 5.1 ($\log(M_{\odot})$) with a dispersion of 0.52 (refs 5, 6). The number of clusters formed in the simulation (1,160) was chosen so that the number with $m_V \leq 22.5$ matches that in NGC4038/4039. Other assumptions used in the simulation are the most natural for globular clusters in the NGC4038/4039 system and do not represent a fit to the data. The sharp drop-off at fainter magnitudes is due to the limiting magnitude of the observations.

and Schweizer's R_{eff} measurements, causing the disparity with globular cluster sizes.

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1. Brock, T. D. *Thermophilic Microorganisms and Life at High Temperatures* 255–302 (Springer, Berlin, 1978).
2. Starkey, R. L. & Waksman, S. A. *J. Bact.* **45**, 509–519 (1943).
3. Sletten, O. & Skinner, C. E. *J. Bact.* **56**, 679–681 (1948).
4. Darland, G., Brock, T. D., Samsonoff, W. & Conti, S. F. *Science* **170**, 1416–1418 (1970).
5. Segerer, A., Langworthy, T. A. & Stetter, K. O. *Syst. appl. Microbiol.* **10**, 161–171 (1988).
6. Ouchterlony, O. *Prog. Allergy* **VI**, 30–154 (1962).
7. Sturm, S., Schönfeld, U., Zillig, W., Janekovic, D. & Stetter, K. O. *Zentralbl. Bakt. I Abt. Orig. C* **112**, 12–15 (1980).

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The theologies of science

Walter Gratzer

The Third Culture: Beyond the Scientific Revolution. Edited by John Brockman. *Simon and Schuster: 1995. Pp. 413. \$27.50.*

WHEN President Lincoln was assassinated, Matthew Arnold, that pillar of Victorian cultural values, was excited to hear that the murderer had shouted in Latin as he leapt from the stage. This persuaded Arnold that there was hope for America. John Brockman, literary agent and purveyor of science to the masses, believes that the new culture — that is to say science — has vanquished and supplanted the old and that America is its heartland, “the intellectual seedbed for Europe and Asia”. More, “the emergence of the third culture introduces new modes of intellectual discourse and reaffirms the pre-eminence of America in the realm of important ideas.” Well, fair enough and *touché*, for Brockman is perfectly entitled to pay the Europeans back for their insufferable assumptions of superiority in happier times. He may not be as uncivil as Ruskin — “England taught the Americans all they know of speech or thought, hitherto. What thoughts they have not learned from England are foolish thoughts; what words they have not learned from England unseemly words” — but the message is plain.

The message, on the other hand, is not new. Who, would you guess, opined that “what art was to the Ancient World science is to the Modern”? It wasn’t Brockman or C. P. Snow, nor yet Jacob Bronowski, but Benjamin Disraeli. Forty years on, and H. G. Wells and his cronies were spreading the word that only Men of Science were qualified to run the world as it then was, and that the governing classes should bow to their successors if they knew what was good for them. A generation later we had Snow, and now John (the Baptist) Brockman with his proclamation of the new order. He believes that science books have only now achieved a proper popularity and that the big sellers (most prodigiously of course Stephen Hawking) are not merely bought, but are read. I think he is wrong on both counts; as to how many of the three million buyers got further than ten pages into Hawking, I have only anecdotal, though (to me) compelling, evidence; but the first printing of *The Origin of Species* sold out in a twinkling, Einstein’s popular book on relativity stayed in print for decades and in Britain the blue Pelican editions of Haldane, Julian Huxley, Waddington and many others were eagerly seized on.

So what of Brockman’s showcase of the brave new culture? It is in its way captivating. Brockman has assembled two dozen

voluble scientists, most of them with a commitment to popular exposition, and in interviews, from which the editor’s prompts have been expunged, gives them their head. When they have had their say, their *confrères* are invited to comment. Some of the contributors have by now become rather predictable. There are no new revelations in the opinions aired by Stephen Jay Gould and Richard Dawkins, but it is still quite fun to see them belabour each other with bladders on sticks — though the psychologist Nicholas

“... mainly the tributes go something like this: ‘Jack is one of my most cherished friends. No-one can have a higher opinion of him than I do and I think he is an unctuous, boneheaded little creep.’”

Humphrey suggests they would do well to desist from such unseemly brawling (“Richard is wrong because. . .”, “Steve doesn’t understand this. He keeps going on about. . .”). The tone is that of the debates between the mediaeval schoolmen, for the arguments derive mostly from pure reason and certainly not from experiment. Lynn Margulis sinks her teeth in Dawkins’s ankle (“... unlike the rest of us, *he* values the truth. The inference of his statement simply exposes his solipsism”) and Daniel Dennett in Gould’s (“He tried it on, he tried it pretty hard, and when it didn’t sell he backed off”). There are of course alliances, manifested by exchanges of shamelessly fulsome flattery, but mainly the tributes go something like this: “Jack is one of my most cherished friends. No-one can have a higher opinion of him than I do and I think he is an unctuous, boneheaded little creep.”

I wonder also what impression something like the following might make on the scientifically innocent reader in search of enlightenment: “In medicine, chronobiology is regarded as the new wave in treatment of any kind of disease, because you have to be able to tune into the system at the right phase, at the right time.” Thus Brian Goodwin, and the vein is inexhaustible: “Mathematics is a tool for exploring generic forms, natural forms, and a way of looking at their stability and their dynamics and their change and so on. But you have to couple that with the internal, experiential aspect of creativity. That is what the postmodern science of

qualities is about.” This is deplorable stuff and Dawkins for one does not miss his opportunity: “We thought when he moved to the Open University, where they’re all crazy, then Brian Goodwin would switch back and become sensible. I don’t think that’s happened.”

There is a rather repugnant sense of autointoxication in too many of these acts of self-revelation. Bertrand Russell famously observed that the conviction that one’s work was extremely important was a sign of approaching madness, for which he would urge early psychiatric treatment. Order is restored to some extent by the ever-admirable Steve Jones. He speaks about his own field of snail genetics, which he holds to be “a microcosm of evolutionary biology at its worst. Its literature is filled with the great vagueness of evolution — with words that, when you deconstruct them, are like shovelling fog; they don’t mean much. ‘Coadaptation’, ‘adaptive landscape’, ‘punctuated equilibrium’ — what I sometimes think of as theological population genetics. They’re words that don’t help at all when you’re trying to decide what experiment to do next.” The missiles are well aimed. Jones, alone among the participants in this striptease show, also has a heartening line in self-deprecation. “I can console myself”, he says, concluding his brief dissertation, “with the thought that I’m one of the top six snail geneticists in the world, out of a field of perhaps half a dozen.”

I turned with high hopes to the physicists, mathematicians and psychologists, who occupy the second half of the book, but found there even more dissension and acrimony. Roger Schank, who ponders the nature of cognition, devotes much of his piece to a denunciation of Noam Chomsky (who was not of the party, and will probably bear the blows with equanimity): “Noam Chomsky represents everything that’s bad about academics” and engages in “nothing less than intellectual dirty tricks”. Nor has Schank much charity in his heart for Roger Penrose, who *is* on the platform (“It’s very sad that people write books about subjects they don’t understand”). Or consider Francisco Varela’s thoughtful appreciation of Marvin Minsky: “. . . he’s a pain in the ass, an arrogant son of a bitch”. Minsky prudently keeps his counsel.

Varela comes through as a mystic — the man with staring eyes and a theory on the whereabouts of Christ’s foreskin or King Arthur’s round table, whom you edge away from in the saloon bar. Here he goes, sounding off on the subject of AIDS: “AIDS is a dramatic case of the deregulation of this coherent emergent property, much like ecological dysfunctioning.” Ah, so!

Others are equally obscure. Penrose starts well enough, becomes opaque and then goes onto microtubules and off the

rails, when searching for the seat of consciousness. Too much of the biological, or rather biomathematical, thinking in fact is reminiscent of the trends of 40 or so years ago, when theoreticians of various stripes surged into biology with talk of fast excitation processes (by way of the first discovered ordered structure, the α -helix) in the brain, low-lying excited (semiconductive) states in proteins, and so on. None of this connected with biologists, going about their daily business. Jones goes further: grappling with concepts as elusive as consciousness, he suggests, is simply not the business of scientists. As Lord Kelvin put it, only that which can be measured and expressed in figures counts as science.

In the midst of all this, Steven Pinker on language comes as a draught of cold, clear water, comprehensible, stylish and stimulating. The four cosmologists, Martin Rees, Alan Guth, Lee Smolin and Paul Davies, Big-Bangers all, are interesting and convey the impression of a serene authority; I was troubled only by the recollection of Lev Landau's adage that cosmologists are often wrong but never in doubt.

There are some engaging reflections from Doyne Farmer, a hero of Thomas Bass's absorbing book *The Newtonian Casino*. A footloose, quintessentially American figure, he has moved from his attempts to break the bank at Las Vegas, through academic physics, to the creation of a company dedicated to making money on the financial markets with the aid of computer learning algorithms. Keynes of course made a packet for his college with no such technological aids. Daniel Hillis is another visionary who has set up a concern dedicated to developing a "connection machine" — a computer that will simulate thought. Stuart Kauffman believes he will be able to develop polymers, based on the biological molecules that we know, which will catalyse any reaction, make vaccines to treat any disease and fulfil a few other modest aims; he has already, he tells us, taken out a patent, so it must be just around the corner, no? Well, as Dr Johnson observed, a man is seldom so harmlessly occupied as in making money.

The danger of Brockman's extraordinary conflation, it seems to me, is that people who do not know much about science will take it for a true picture of what scientists actually do and how they behave towards each other. It is no such thing. You would not guess from anything you glean here that genes make proteins and do not merely ride around in the individuals of a species. You would remain in the dark about molecular genetics, molecular structure, neurobiology, the mechanisms of development and growth and the nature of diseases — there is nothing in short about any of the most striking technical and intellectual advances of our

time: Brockman has invited the reader to a dinner of horseradish and mustard without the roast beef. Chemistry is not represented and most working physicists — in fundamental particles, say, or solid-state — would not find in these pages any physics that they recognize. But as a tour around the wilder fringes of scientific enquiry it is undoubtedly a valuable and engrossing document. With some exceptions, I would not want to be stuck for long in a lift with members of this *galère*, but I willingly admit that I was seldom bored. Brockman may besides have done them (and us, the readers) a service in finally, if unwittingly, showing Caliban the mirror. □

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Beyond natural selection?

Jonathan Cooke

Epigenetic Inheritance and Evolution: The Lamarckian Dimension. By Eva Jablonka and Marion J. Lamb. *Oxford University Press: 1995. Pp. 346. £29.50, \$49.95.*

THERE is currently a rather bland feeling of consensus, at least over large issues in biology. So it is refreshing to find a book within the subject (as opposed to one about it, by sociologists) that begins by predicting profound irritation in its readers — a partially correct prediction in my case but, I suspect, for quite unintended reasons. Despite the introductory chapter tracing apparent links between ideas and historical figures, I am convinced this book is wrongly subtitled. Only when I began to re-read it without reference to Lamarck did my irritation die away. It is surely central to Lamarck's idea that what is passed on to the descendants is created by adaptive 'effort' in the ancestors' struggle to survive. Few if any of the phenomena discussed here conform well enough to that original conception of "the inheritance of acquired characters" to be candidates for even neo-Lamarckian mechanisms.

The authors clearly believe that natural selection of random changes in nucleic-acid sequences is the primary agent of evolution; as they neatly put it, the initial evolution of adaptability itself seems explicable only in this way. But they believe that, once arrived at, various supplementary mechanisms of adaptability (as opposed merely to adaptations) have acted in a sort of bootstrapping process to increase tempo and diversity in evolution-

ary change and in speciation itself. These mechanisms mostly involve what they term 'directed' changes, incurred as responses to environmental challenge, which can be propagated as intrinsic alterations of phenotype in subsequent generations. The authors go on to argue that such alterations, if of sufficient near-genetic stability, act as forces redirecting natural selection — truly genetic selection can act only by altering the incidence or stability of phenotypes that actually occur — until the new characteristics have been 'genetically assimilated'. Such assimilation of initially environmentally induced characters is understandable without unorthodox assumptions, and this is duly reviewed. The novel notion is that the quasi-stable 'epi-mutations', currently seen as a collection of arcane and marginal phenomena, may really be a widespread and (for ideological reasons within current neo-Darwinism) underrecognized force in evolution.

The authors provide a great service in surveying a wonderful collection of observations for which we would love to have molecular explanations, and the book is engrossing for this reason as well as for its thesis. But I find their coy use of the key term 'directed' unsatisfactory. Early on it is used to define quasi-genetic changes that, although not random in informational terms (as are most true mutations), must not be assumed to be adaptive but merely in some sense specific in relation to their environmental causes. Yet this is often belied by the authors' ideas about how mechanisms of rapid adaptability might have arisen using this capacity for 'directed' change as raw material.

Another questionable concept is that of (environmentally imposed) 'stress'. It is hard enough to define psychic or even physiological stress, let alone the evolutionary or genomic varieties, and professional use of the term seems best left to engineers. Then, paradoxically, when the authors discuss gene-methylation changes that survive gametogenesis in mammals, they fail to state explicitly that here could be a mechanism for the closest approach to Lamarckian inheritance we are likely to see. Genes may have their transcription rate initially changed as a truly adaptive response to environmental stimuli, whereas methylation status probably results from, and then stabilizes or perpetuates, probabilities or rates of gene transcription initially set up by other means.

Much selection in early evolution must have been for genomes that could establish large clones in the widest variety of circumstances, with little need to survive the complexities of sexual recombination or 'development'. Mechanisms allowing propagation of induced steady states of gene activity (as in yeasts), structural inheritance (as in ciliates) and the apparently environmentally directed but true

DNA mutations seen in bacteria, would thus be important if they were possible. Recent information helping us to verify and understand such phenomena is well reviewed in the book. But the demands of multicellular life are different, and here the authors' argument relies on the proposition that subgenetic modes of cellular heredity, which we today associate with the mechanism of differentiation and development (hence epigenesis), can be transmitted across generations. The authors propose that such mechanisms were already in place as adaptability enhancers in unicellular eukaryotes, and may actually have allowed development — that is, multicellularity — to evolve.

Selective methylation has already been mentioned, but much is also made of the possibilities from changes in large-scale linear organization of DNA — that which may situate individual genes in regions of particular overall base composition or in regions that are constitutively or conditionally (cell-type-dependent) late-replicating and unavailable for transcription. But surely this organizational level of DNA, which can indeed influence the timing of gene activity, is part and parcel of the selectable mechanism of true DNA heredity. The genetic and at least this part of the epigenetic mechanism are one.

'Mutation' frequency for large-scale sequence re-ordering may well exceed that for the gene-coding and local control-sequence details of traditional molecular genetics, and this may have accelerated evolution. But that does not itself make the genome, as the authors put it, a "flexible, responding system" of the organism. Higher plants differ from much of the animal kingdom in throwing germ cells literally off the ends of their entire somatic lineages, rather than sequestering them early on from the need to display a functional phenotype and face environmental challenge in the soma. Significantly, I was more impressed with the evidence among plants than that among animals for (conceivably 'directed') epimutations as underlying the phenomenon of adaptive ecotypes, and perhaps rapid speciation.

As Jablonka and Lamb virtually say, their book in effect asks: has our relative ignorance of developmental mechanisms during most of the neo-Darwinian era profoundly distorted our understanding of evolution? I was left with the feeling that on the whole it may not have, even though as a developmental biologist I should have enjoyed being persuaded that my discipline is absolutely central. But whoever is more correct, the book is an extremely worthwhile one to have written and to have read. □

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On growth and form

Lionel G. Harrison

The Algorithmic Beauty of Sea Shells.

By Hans Meinhardt. Springer: 1995. Pp. 204. DM78, \$49.95, £34.

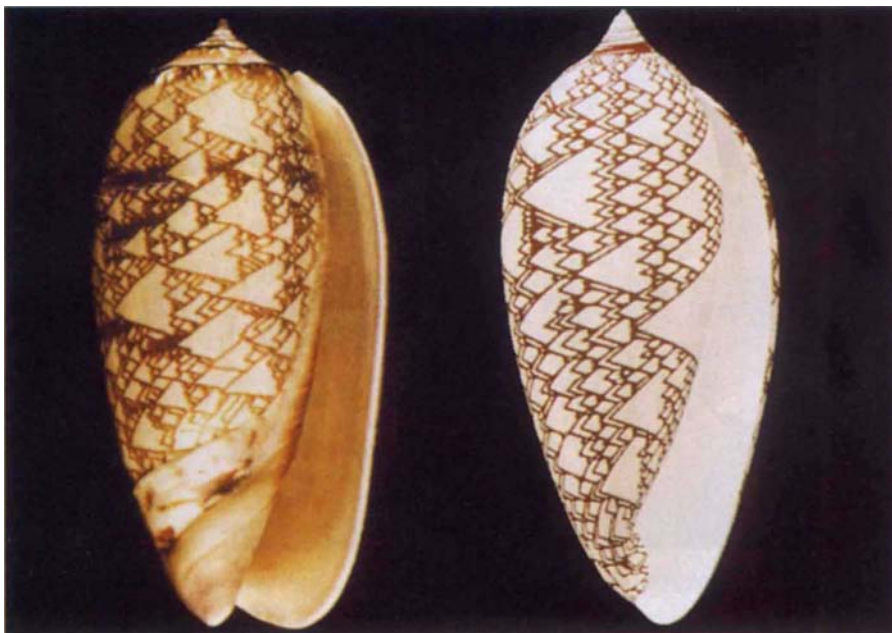
HANS Meinhardt has achieved the remarkable feat of producing a book worthy of the coffee-table yet replete with partial differential equations. His topic is the diversity of pigment patterns on tropical sea shells. A mollusc enlarges its shell only at the edge; pigment is usually laid down by this essentially one-dimensional growth zone. The pattern on the shell's surface is therefore a spacetime diagram of the cells in the growth zone that are active in pigment production. If these remain fixed in position along the growing edge, each active region draws a line perpendicular to that edge. If the whole growth zone is simultaneously switched on and off in oscillatory fashion, lines parallel to it result. Diagonal lines arise from active regions that move along the growing edge like travelling waves.

How are the stationary or moving locations of these living paintbrushes controlled? Like me, Meinhardt has been since the early 1970s devoted to the concept that living pattern is often a manifestation of dynamics that couple reaction kinetics to diffusive transport. He is single-minded about this. He pursues reaction-diffusion schemes relentlessly, hardly ever admitting the existence of other possible dynamics, such as mechanochemistry. But this is a good research strategy. Reaction-diffusion equations contain the essence of how any kind of nonlinear dynamics can generate

pattern and are the most widely elaborated form of these dynamics since Alan Turing proposed them in 1952. Twenty years later, Alfred Gierer and Meinhardt devised their equations, at first without knowledge of Turing's work. But their models are an important subset of the very broad category of Turing dynamics.

Sea-shell patterns are not limited to parallel lines. They include arrays of V-shaped branchings, drop-shaped unpigmented patches, and separated fragments of pattern as in the well-named *Lioconcha hieroglyphica*. Colour examples give the book its pictorial richness. To model these, Meinhardt has started from the essential minimum of two mutually interacting diffusible morphogens, and for some patterns has increased the complexity of the model to three such morphogen pairs. His personal method emerges clearly: qualitative arguments, expressed in words, on the effects of changing parameter values or introducing new dynamic features; continual testing of these ideas by computer experiments; and nothing much of parameter spaces and other mathematical analysis.

What should this elegant and impressively successful modelling signify to the developmental biologist? In 1956, C. H. Waddington dismissed Turing models to the nether-world of "the quasi-periodic dappings and mottlings which often fill up relatively unimportant spaces". Sea-shell patterns, commonly hidden in life and evolutionarily useless, are quite possibly in that category. But I see their means of production as the idling of that same motor that, at full power, drives the formation of the embryonic body plan. Meinhardt's work through the 1970s and 1980s clearly shows the same belief; most of it has been on crucial developmental



Shell shock — striking similarity of a photograph and computer model of *Oliva porphyria*.

From *The Algorithmic Beauty of Sea Shells*

processes, not superficial features. For anyone inclined to the dynamic paradigm of pattern formation, Meinhardt's book, although inadequate as a historical review, is a fine introduction to thinking about dynamics that is, into the bargain, readable and beautiful. It is accompanied by a diskette containing an easily run demonstration program to reproduce all

the computed figures in the book, and full source codes to allow readers to change parameter values and get the feel of dynamic modelling. □

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The sea is a very big place

Paul F. S. Cornelius

Monsters of the Sea: The History, Natural History, and Mythology of the Oceans' Most Fantastic Creatures. By Richard Ellis. Knopf/Hale: 1994/1995. Pp. 429. \$30, £22.

SCIENCE should be fun, or scientists would be fools to do it. But its integrity is never tested more rigorously than in studies in which all the data come from non-scientists. Although scientific method does not allow belief, much reasoning on the scientific shop-floor follows hunch and intuition. 'Healthy' disbelief must often be pragmatically replaced by agnosticism, or progress becomes hampered. Investigation of monster stories requires just this kind of approach.

Richard Ellis brings cold scientific light to bear on numerous monster reports. Thus it is disappointing to learn that, as revealed last year, the main basis of the Scottish 'Loch Ness monster' stories was a hoax started by newspaper reporters. But all is not lost for monster fans. I once heard a non-zoologist ask the late Sir Frederick Russell, one of the sea's shrewdest students, why an enormous jellyfish from the deep Atlantic that he

had described (in *Nature* **184**, 1527; 1959) had not been recognized earlier. He replied simply: "The sea is a very big place". Thankfully it still is, and a few of its huge life-forms may be still undiscovered.

Ellis's detailed and interesting account is an impressive compilation. It will satisfy readers from a wide spectrum, from lay people to researchers. Although it is not essential reading, every marine biological library and larger public library should have it. It will be useful also to those interested in the phenomenon of mass human self-deception, including psychologists and media moguls. Some in the last two categories, and sadly a few zoologists, have lined their pockets fuelling belief in monster myths, and they may not welcome this honest book.

Ellis criticizes himself for harshly analysing at length Jules Verne's fictional monsters, even though Verne had artistic licence on his side. Jacqueline Goy of the Muséum National d'Histoire Naturelle in Paris tells me that in fact a nineteenth-century French zoologist, H. Milne Edwards, was largely responsible for some of Verne's fantastic monster creations, making, for example, the giant squid in *Twenty Thousand Leagues*

Under the Sea entertainingly plausible.

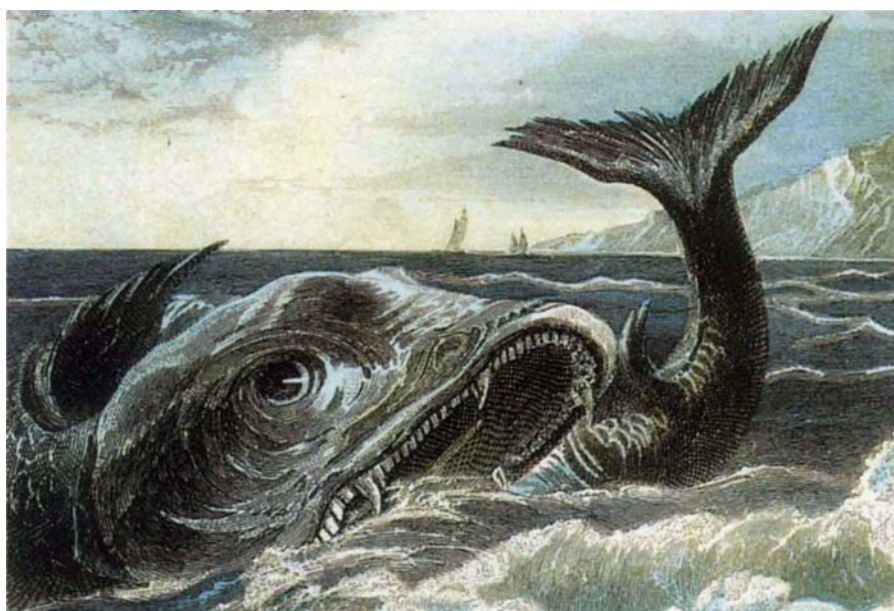
Ellis's straightforward enquiry successfully relates most 'monster' beliefs to known species. One therefore feels that among a few unexplained reports there may truly lurk an unknown animal. The main sections concern the Loch Ness monster (now at last getting its come-uppance), sea serpents (mainly based on giant squid, oarfish, and large whales and sharks once commoner in populated regions), mermaids (mainly manatees) and a special treatment of sightings of giant squid, including an impressive tabulation of records of this, or these, still poorly known species.

Scientists will feel at ease with the book because it maintains its agnosticism throughout. Some other volumes on the subject, such as Michael Bright's *There Are Giants in the Sea* (1989), although similarly painstaking compilations, are for believers who want to become convinced. To read Bernard Heuvelman's classic *On the Track of Unknown Animals* (1958) one might think that the world were full of undiscovered and spectacular vertebrates; but Ellis's book implies that what actually abounds is human misconception and unjustified belief.

What undiscovered monsters might there yet be? A carnivorous, perhaps deep-sea, shark (*Carcharodon megalodon*), three times the length of the great white shark, is considered to have become extinct shortly before historical times but might conceivably be found alive. After all, runs the irrefutable argument, the huge megamouth shark *Megachasma* was not found until 19 years ago. There are still vast tracts of the world, and especially of the sea, where scientific expertise is sparse. Last year I was able to add several species of jellyfish the size of dinner plates to the faunal list of Australia, some within sight of large towns. Perhaps giant squid with eyes much larger than dinner plates are right now fighting for their lives with enormous sperm whales 3,000 feet below the ocean surface in terrifying struggles that mankind has still to witness — great battle-scars are occasionally seen on sperm whales, which habitually eat squid. Ellis considers the most likely monster we will yet discover is a giant deep-sea octopus with an arm-spread of 200 feet; but, right to the end, he cautiously avoids persuasive speculation.

The sea is indeed a big place and some awesome animals live in it. Ellis's book weeds out the myths, leaving an account of genuine monsters that, because they really exist, are more impressive than their fictional counterparts. Like all good science, it is undeniably fun. □

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Making waves — detail from *Jonah and the Whale* by W. French, after J. Vernet.

Extraction of mid-ocean-ridge basalt from the upwelling mantle by focused flow of melt in dunite channels

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Like residual peridotites from mid-ocean ridges, peridotites from the mantle section of the Oman ophiolite are far from equilibrium with mid-ocean-ridge basalt (MORB). By contrast, dunites from Oman are close to equilibrium with MORB, indicating that they were conduits for focused melt flow. Formation of dunite conduits by porous flow is sufficient to explain extraction of MORB from the mantle, and fracture mechanisms may not be necessary in this process.

MORB forms by partial melting of adiabatically decompressing mantle peridotite beneath spreading ridges (see, for example, refs 1–3). Nevertheless, MORB is not in equilibrium with residual peridotite (harzburgite and lherzolite). For example, MORB is not saturated with the mineral orthopyroxene (opx) at Moho pressures, whereas opx is a major constituent of residual peridotites. Liquids parental to MORB are saturated with opx only at pressures greater than 8 kbar (for example, refs 4, 5), more than 15 km below the Moho. In addition, MORB is not in trace-element equilibrium with most abyssal peridotites (residual mantle samples dredged from the mid-ocean ridges)^{6,7}. Instead, MORB is interpreted as a mixture which preserves geochemical evidence for equilibration with mantle peridotite over a range of pressures, much higher than the pressure at the base of the crust^{8,9}.

A polybaric, near-fractional melting process can account for the observed composition of MORB and residual peridotites^{6–10}. To produce large fractionations between light and heavy rare-earth elements, and between other elements such as Ti/Zr, Lu/Hf and U/Th, small melt fractions must be efficiently segregated from their sources and mixed to form MORB. To preserve these fractionations, and to produce disequilibrium between melt and opx at low pressure, melts must be transported to the crust without re-equilibration with surrounding peridotite. Diffuse porous flow of melt through residual peridotite from the source to the crust would lead to extensive chemical reaction, bringing the liquids close to low-pressure opx saturation^{11,12} and decreasing or erasing trace-element fractionation^{10,13,14}. Thus, focused flow of melt in spatially restricted conduits is required for MORB extraction from the mantle.

Two end-member mechanisms for focused extraction of MORB have been proposed: (1) development of hydrofractures extending from near the bottom of the melting region to the surface¹⁵ and (2) formation of high-permeability channels by dissolution of solid phases during focused porous flow of adiabatically ascending melt^{12,16}. The present study provides evidence that constrains the nature of conduits for melt extraction beneath mid-ocean ridges, and the mechanisms by which they form. We present observations on dunites (rocks composed of >90% olivine) in the mantle section of ophiolites. Phase equilibria and field evidence, from Oman and elsewhere, show that dunites form by dissolution of pyroxene from peridotite (rocks with >40% olivine and >10% pyroxene) in magma migrating by porous flow. Previous petrologic and geochemical studies focused on dunites which replace lithospheric mantle peridotites, whereas dunites in Oman formed in the adiabatically upwelling

asthenosphere beneath a spreading ridge. New major- and trace-element data on the compositions of minerals in the mantle section of the Oman ophiolite indicate that dunites are conduits for extraction of MORB from the mantle. Together, these lines of evidence indicate that MORB extraction from the upwelling mantle involves focused porous flow through dunite channels.

Geology and petrology of dunites

Figure 1 illustrates the contact relationships of residual peridotite and dunites in the Oman ophiolite, compiled from previously published work. Dunites comprise 5 to 15% of the exposed mantle section in Oman^{17,18}. They preserve sharp, irregular contacts which are locally discordant to banding and crystallographic lineation in the harzburgite (see, for example, Lippard *et al.*¹⁸, p. 53). Because harzburgites and most dunites now have a subhorizontal foliation, parallel to the palaeo-Moho, we know that both were transposed by corner flow beneath a spreading ridge. Thus, the transposed dunites were formed in upwelling mantle beneath a spreading-ridge axis. Here we further constrain the physical and chemical conditions which led to their formation.

Dunites within the mantle section of ophiolites have been interpreted as (1) residues of very high degrees of partial melting (for example, ref. 19), (2) olivine 'cumulate' dykes, that is fractures filled with olivine precipitated by ascending melt (for example, refs 20, 21), and (3) products of reaction between olivine-saturated magma and pyroxene-bearing rocks^{11,12,15,22–26}. A residual origin for dunites is unlikely, as complete removal of pyroxene from mantle peridotite by melting requires very high degrees of partial fusion (>40%), at mantle potential temperatures much higher than those beneath mid-ocean ridges (see, for example, the summary of experimental results in ref. 3). A cumulate origin is also unlikely, as adiabatically ascending melt in a crack is not saturated in any solid phase, including olivine (for example, ref. 27). Under lithospheric conditions, conductively cooling melt in fractures may gradually stagnate to form dykes. However, dykes are rarely if ever composed only of olivine, as olivine is very different in composition from any natural silicate melt. Also, the observed ratio of Mg/(Mg+Fe²⁺), denoted 'Mg number' or 'Mg#', is generally very similar in olivine from dunites and associated mantle peridotites¹¹. Compared to olivine in residual mantle peridotite, olivine in residual dunite should have higher Mg#, and olivine in cumulate dunite should have lower Mg#.

Thus, few if any dunites are residual or cumulate. Instead, most dunites in the mantle section of ophiolites form by dissolu-

tion of pyroxene, with concomitant precipitation of olivine, during porous flow of olivine-saturated melt through mantle peridotite^{11,12,15,22-26}. Contact relationships indicate a replacive origin for dunites (Fig. 2). A replacive origin also can explain the similar olivine Mg#s in dunites and host peridotite¹¹. Formation of replacive dunite occurs where relatively high-pressure partial melts of the mantle react with peridotite at a lower pressure^{11,12}. This process can also occur at constant temperature and pressure, but is likely to be more common and extensive in the adiabatically ascending mantle. In open systems where migrating basalt reacts with the adiabatically ascending mantle, liquids remain saturated with olivine. This is different from the adiabatic ascent of melt in an open crack, owing to the effects of maintaining solid-liquid equilibrium. Formation of replacive dunite on an adiabat will result in an increase in the liquid mass^{11,12} and therefore an increase in porosity. Increasing porosity, and the relatively higher permeability of dunite compared to pyroxene-bearing rocks^{28,29}, leads to higher permeability in the dunite. Regions with initially high permeability have a high melt flux, which leads to more rapid dissolution in these regions, producing even higher permeability. This positive feedback mechanism (the 'reactive infiltration instability'³⁰) can produce elongate dunite channels in the mantle parallel to the melt flow direction^{12,16}.

In addition to forming purely as a result of porous, reactive flow, dunites form in ductile shear zones³¹ and in porous reaction zones around propagating cracks¹⁵. In the Josephine peridotite, synkinematic dunites in shear zones formed by reaction between hydrous melt and residual peridotite at temperatures less than 1,100 °C, near the brittle-ductile transition in shallow mantle lithosphere. They are nearly vertical, cut a subhorizontal foliation in peridotite, and formed at the same time as near-vertical pyroxenite dykes. Tabular dunites with a medial pyroxenite, such as are common in the Horoman peridotite^{21,32} and the Trinity peridotite²³ may be porous reaction zones around melt-filled fractures.

Ophiolites intrinsically preserve melt migration features formed in conductively cooled 'lithosphere' as well as in adiabatically rising 'asthenosphere'. Pyroxenite and gabbro dykes are observed in the mantle section of many ophiolites. The dykes are generally undeformed, and highly discordant to the high-temperature foliation in residual peridotites, as shown in Fig. 1. Formation of pyroxenes and plagioclase in the dykes is indicative of crystallization from an evolved, low-temperature liquid. Such evolved liquids form from a parental, mantle-derived magma, as a result of crystal fractionation due to conductive cooling. Therefore, we infer that dykes in the mantle section of ophiolites formed off-axis, in oceanic lithosphere with a conductive geotherm. This also applies to dunites and coeval pyroxenites formed in shear zones, and to tabular dunites with medial pyroxenite dykes.

Transposed dunites in Oman are not associated with localized shear zones, and do not have medial pyroxenites. Instead, pyroxenite and gabbro dykes in the Wadi Tayin massif cut transposed dunites at a high angle. Ceuleneer³³ reported that pyroxenite and gabbro dykes are not present in the centre of the Maqsad massif, an area in Oman where structures formed by mantle upwelling beneath a ridge axis are preserved. This important observation supports the hypothesis that dykes form off-axis in lithospheric mantle, and suggests that melt extraction beneath the ridge axis was predominantly by porous flow. In addition, we can infer that adiabatic conditions were maintained nearly to the base of the crust in upwelling mantle beneath the spreading ridge in Oman until magmatism ceased. Localized shear zones and fractures may be rare or absent in the viscously deformable, adiabatically decompressing mantle beneath spreading ridges. By contrast, Aharonov *et al.*¹⁶ show that formation of porous conduits by reactive flow can and will occur in adiabatically ascending asthenosphere. On the basis of this theoretical work, and the field relations and phase equilibrium data just discussed, we conclude that transposed dunites in the Oman mantle section were formed by reactive porous flow in the

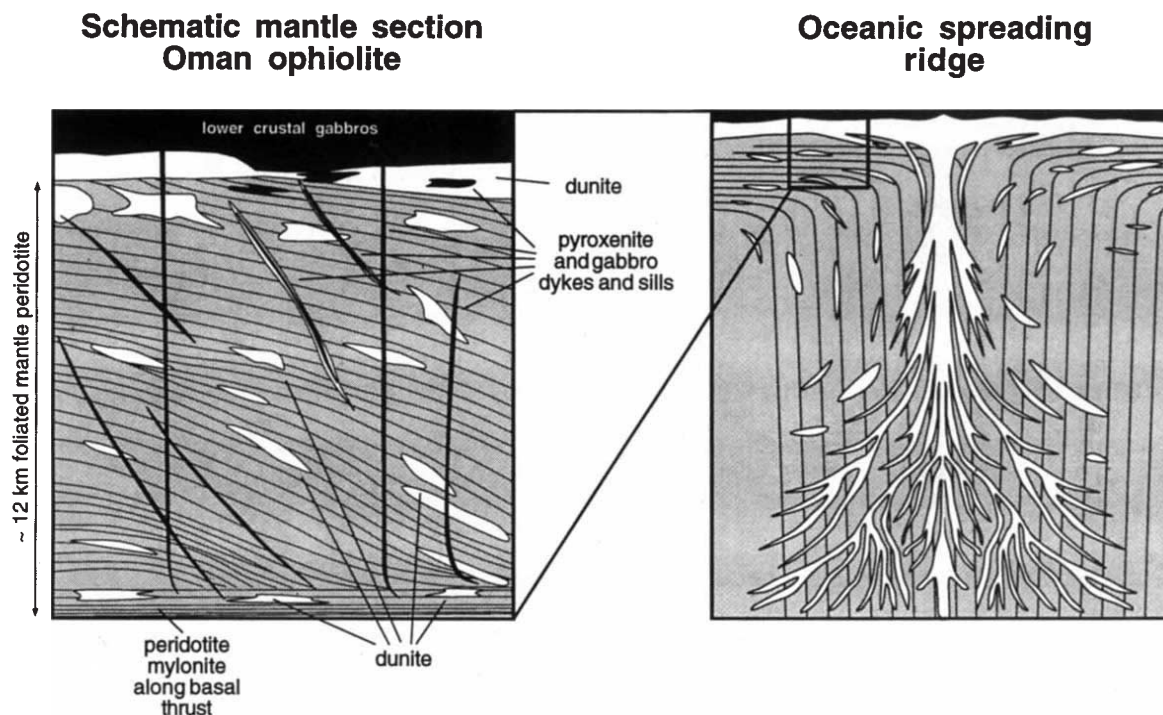


FIG. 1 Left, schematic cross-section of the Wadi Tayin section of the Oman ophiolite, compiled from refs 17, 18, 34 and our own field observations. Right, cartoon illustrating possible ridge-axis geometry of

residual harzburgite, dunite conduits for melt flow and oceanic lower crust. Shading as follows: white, dunite; grey, mantle peridotite; black, pyroxenite and gabbro dykes and sills.

asthenospheric mantle beneath a spreading ridge. In the next section, we present geochemical data which indicate that the dunites were conduits for extraction of MORB from the mantle.

Geochemical data

The Oman ophiolite formed by partial melting in the mantle and accretion of melts to form crust at an oceanic spreading centre^{18,34}. Although they may be different in detail, most of the older volcanics in the ophiolite are similar to MORB. For example, the Geotimes and Lasail volcanic units in Oman are composed of tholeiitic basalts with relatively flat rare-earth element (REE) patterns (chondrite-normalized La/Yb from 0.6 to 1.0), and moderate heavy-REE contents ((10–30) × chondritic)^{18,35}, similar to 'normal' MORB. We infer that processes similar to those which form MORB at oceanic spreading centres operated in the formation of the Oman ophiolite. (Younger suites of volcanics in Oman are distinctly different from MORB^{18,35} and may have formed during or after subduction associated with ophiolite emplacement³⁴.)

Samples analysed in this study are from a traverse across the mantle section (15–20 km thick) in the Wadi Tayin region of the Oman ophiolite¹⁷. Residual peridotites in the section are mostly harzburgites with about 75% olivine, 20% opx, <5% clinopyroxene (cpx), and a few per cent spinel. Cpx in these samples occurs as subhedral aggregates, as small grains associated with opx porphyroblasts, and as undeformed, interstitial grains. Cpx is generally unzoned, but represents a recrystallized, polygenetic mixture of residual material, cpx exsolved from opx, and perhaps cpx crystallized from migrating melt.

Dunites have >95% olivine, 2–3% spinel and <1% interstitial cpx. Cpx in dunites occurs exclusively as cusped grains along olivine triple grain boundaries. A sample from a small chromitite body within dunite has >85% euhedral chromite, and <15% interstitial olivine + cpx. (As in most ophiolite mantle sections, chromitite occurs only within dunite in Oman.) As in the dunites, cpx in the chromitite occurs at triple grain boundaries. Adiabatically ascending basaltic liquids are not saturated in cpx. Also, cpx is not a product of most reactions involving adiabatically ascending mantle peridotite and basaltic liquid^{11,12,26}. Cpx in dunites must have formed by partial crystallization of cooling, migrating liquid as the dunites passed out of the upwelling asthenosphere, into a conductive thermal regime. Basalt and cpx do not have the same composition, so that crystallization of basalt in a closed system forms plagioclase, plus olivine and/or opx, as well as cpx. Because neither plagioclase nor opx is observed in our dunite and chromitite samples, we are confident that the cpx formed in an open system. It did not form as a product of complete, closed-system crystallization of 'trapped liquid'.

There is a first-order difference between dunite and harzburgite in both cpx and spinel composition. Cpx and spinel in samples from the Wadi Tayin massif of the Oman ophiolite have been analysed by ion and electron microprobe. Where possible, 3–5 crystals of each mineral were analysed in each sample. Ion-probe techniques are summarized elsewhere³⁶. Dunite and chromitite cpx have a slightly light-REE-depleted, chondrite-normalized pattern (Fig. 3). Harzburgite cpx from the Wadi Tayin section is strongly depleted in light REE, similar to the most depleted cpx in abyssal peridotite^{6,7}. Within-sample and within-group variations are much smaller than differences between dunite and harzburgite groups.

Liquid compositions in equilibrium with cpx crystals can be calculated using experimental cpx/basalt distribution coefficients. We used those of Hart and Dunn³⁷, but virtually any published values could be used with similar results. Calculated liquids for cpx in Wadi Tayin harzburgite are strongly depleted in light REE, and thus are very different from MORB. Like abyssal peridotites, Oman harzburgites have been depleted by near-fractional partial melting. In detail, calculated liquids for harzburgite cpx are enriched in La and Ce compared to Nd.

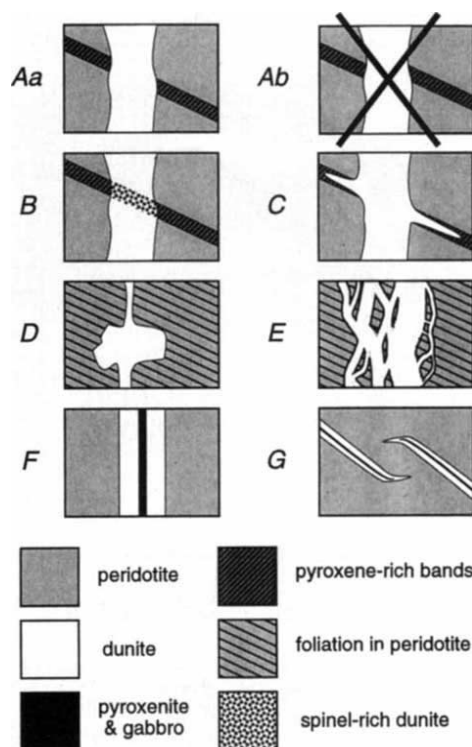


FIG. 2 Summary of contact relationships of dunites in the mantle section of ophiolites. For specific examples and additional references, see refs 11, 12, 15, 17–26, 31–34, 46, 50, 52 and 53. We interpret the relations illustrated in A–E to indicate a replacive origin for dunites, involving dissolution of pyroxene and precipitation of olivine (± spinel) by liquid migrating by porous flow. Dunites with contact relations as illustrated in F and G are probably replacive, but may have formed in porous reaction zones around melt-filled fractures. A, Dunite that cross-cuts pyroxene-rich bands in peridotite does not displace the bands, as in Aa. If dunites were dykes filled with olivine, bands in the wall rock would be displaced, as in Ab. B, Relict trains of spinel where dunite cross-cuts pyroxene-rich bands. Some spinel is present in the original bands; additional spinel may be precipitated during incongruent dissolution of pyroxene. C, Locally, pyroxene-rich bands are selectively replaced by dunite without evidence for dilation by dyke injection. D, Widening features, where narrow dunites enter and leave a larger, rounded region, do not show dilation of foliation in surrounding peridotite. E, Anastomosing dunite includes 'islands' of relict peridotite which have not been rotated or displaced relative to the surrounding peridotite. F, Medial pyroxenite and/or gabbro is observed in some tabular dunite bodies. G, Curving tips in tabular dunite with medial pyroxenite, observed in the Trinity peridotite (A. Rubin, personal communication), suggest that these dunites formed in a porous reaction zone around and ahead of propagating fractures.

At the very low concentrations of La, Ce and Nd in these cpx, the data may be inaccurate. If the relatively high La/Nd and Ce/Nd are real, such 'U-shaped' REE patterns may be attributed to reaction between relatively small amounts of liquid and much larger amounts of residual peridotite^{32,38,39}, or to addition of small amounts of 'trapped liquid' to residual peridotite^{40,41}. Nevertheless, because harzburgites preserve light-REE depletion produced by near-fractional partial melting, migrating liquids that equilibrated with these rocks would have had much lower light/heavy REE ratios than MORB. Thus, as stated previously, pervasive, porous flow through residual peridotites cannot be the dominant mode of MORB extraction from the mantle.

In striking contrast to the harzburgites, calculated liquids in equilibrium with cpx from dunite and chromitite have REE slope and abundance similar to MORB and Oman volcanics. In addition, spinels in dunite and chromitite from Oman are similar in composition to spinels in MORB^{42–45}. Specifically, spinels in dunite, chromitite and MORB have higher Cr# (Cr number,

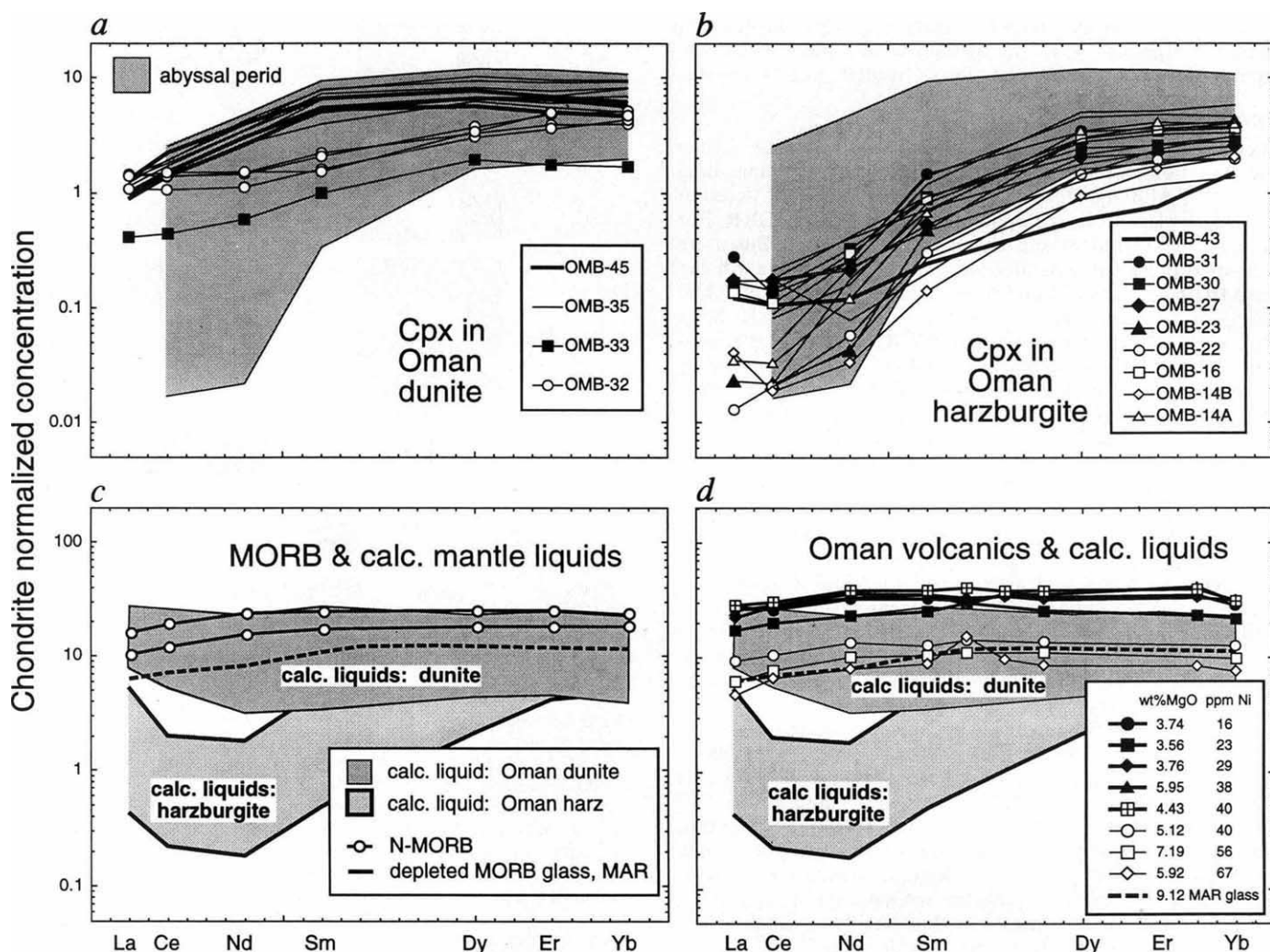


FIG. 3 Rare-earth element (REE) contents of clinopyroxene (cpx) in samples from the Wadi Tayin mantle section of the Oman ophiolite¹⁷, determined by ion microprobe, compared to REE concentrations in abyssal peridotites and mid-ocean-ridge basalts (MORBs). All concentrations are normalized to those in C1 chondrites⁵⁵. These data show that cpx in dunites are close to REE equilibrium with MORB, whereas cpx in harzburgites is not. *a*, REE in cpx from Oman dunites and one small chromitite within dunite (OMB-35) compared to observed concentrations in cpx from abyssal peridotites determined by ion microprobe^{6,7} (shaded region). Several analyses of individual cpx crystals are shown for each Oman sample. All samples were collected by F. Boudier. *b*, REE in cpx from Oman harzburgites compared to abyssal peridotites. Average values are shown for each Oman sample (1–5 cpx points on 1–3 crystals per sample). Lines with symbols are for samples collected

by F. Boudier, lines without symbols are for samples collected from the same section by R. Coleman. Harzburgite OMB-45, transitional between other harzburgites and dunites in its REE slope, is from the crust–mantle transition zone¹⁷. *c*, REE in liquids calculated to be in equilibrium with cpx from Oman dunites and harzburgites (shaded regions) compared with compositions of normal mid-ocean-ridge basalt (N-MORB)^{56,57}, and a low-REE basalt from the Mid-Atlantic Ridge⁵⁸ (MAR). *d*, REE in selected lavas from the Geotimes and Lasail volcanic units in the Oman ophiolite¹⁸. MgO and Ni contents of each sample are given in the figure. MgO and Ni in Oman lavas are low compared to primitive MORB, indicating that the Oman liquids evolved from a mantle-derived melt by crystal fractionation. This fractionation, involving mainly olivine and plagioclase crystallization, would increase the REE concentrations by a factor of <3, and would not modify the REE slopes appreciably.

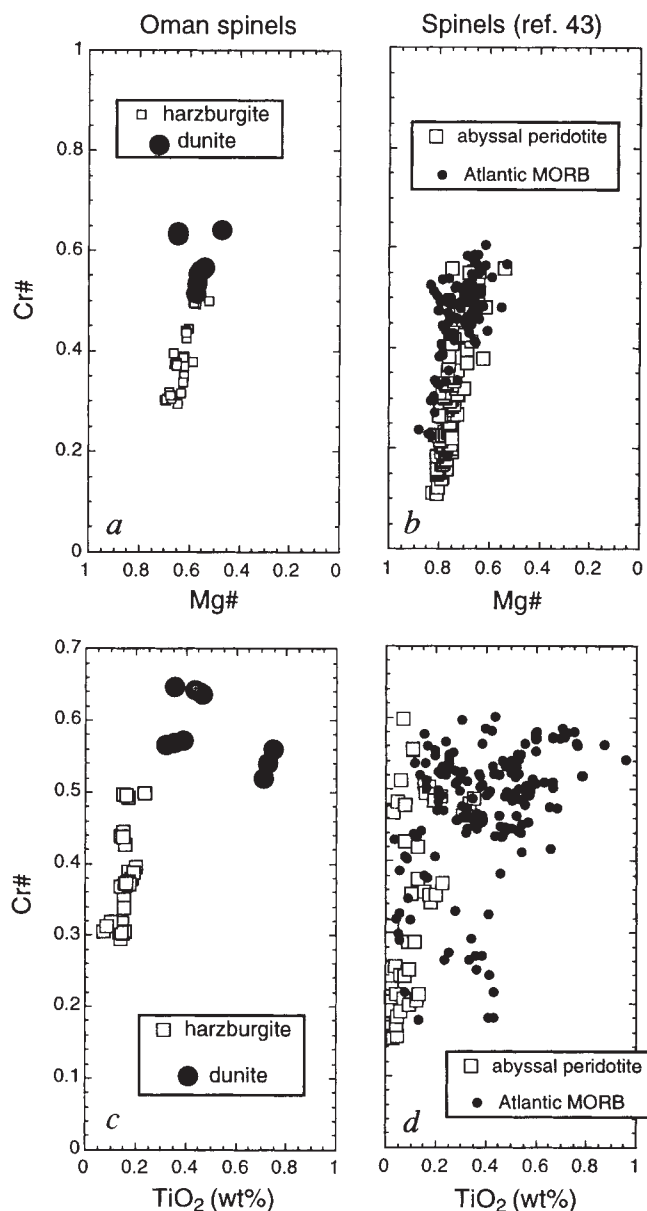
defined as molar Cr/(Cr + Al)), and higher TiO₂ contents, than spinels in Oman harzburgite and in most abyssal peridotites (Fig. 4). Data for Cr# and Ti contents of spinels in Oman volcanics has not been published, but Cr# in spinels from cumulate gabbros in Oman is between 0.4 and 0.6 (refs 18, 46) higher than the values in spinel from most Oman harzburgites and similar to those in Oman dunite. Thus, both cpx REE data and spinel compositions from the Wadi Tayin samples suggest that Oman dunites formed in equilibrium with liquids similar to MORB, whereas harzburgites did not.

Evaluation of geochemical interpretations

Some of the spinels from Wadi Tayin dunites and harzburgites have lower Mg#s than spinels in MORB, as a result of sub-

solidus Fe–Mg exchange between olivine and spinel. This exchange is rapid at temperatures greater than 700 °C, and its effects are observed in almost all peridotite massifs (for example, ref. 47). Spinel Ti contents could also have been increased by metamorphic exchange, in which small amounts of Ti dissolved in olivine at high temperature were added to spinel at lower temperatures. This might affect spinel in dunites more than in harzburgites, as in harzburgites Ti would be added to both spinel and pyroxenes. The Ti contents of coexisting spinel, olivine and cpx from our samples are compared in Fig. 5. Titanium contents are positively correlated in the different phases, and are higher in dunites than in harzburgites. We conclude that Ti concentrations have not been substantially modified by sub-solidus exchange, and

FIG. 4 Cr# (molar Cr/(Cr+Al)), Mg# (molar Mg/(Mg+Fe²⁺)) and TiO₂ contents of spinels in samples from the Wadi Tayin mantle section of the Oman ophiolite, determined by electron microprobe, compared to spinels in abyssal peridotites and mid-ocean-ridge basalts (abyssal peridotite and MORB data from Dick and Bullen⁴³). These data show that spinels in Oman dunites are similar to spinels in MORB, and different from spinels in Oman harzburgites and in abyssal peridotites dredged from the mid-ocean ridges. *a* and *b*, plots of Cr# versus Mg#. *c* and *d*, plots of Cr# versus TiO₂ concentration.



reflect real differences between the liquids which last equilibrated with these samples.

Some high-Cr# spinels in MORB may crystallize from evolved lavas⁴⁴, with elevated Cr/Al produced by low-pressure crystal fractionation of olivine + plagioclase from mantle-derived melt. If this applied to all high-Cr# spinels in MORB, then the similarity in Cr# and Ti between spinels in MORB and Oman dunite would be a coincidence. Figure 6 illustrates compiled data on the composition of associated spinel and olivine in MORB. Olivine crystallized from evolved basalts should have Mg#s less than mantle olivine, which has Mg# ≥ 0.88 . Cr# in spinels is not correlated with Mg# in associated olivine, and is generally greater than 0.4 in samples with olivine Mg# ≥ 0.88 . Trends of decreasing Mg# with increasing Cr# in some lava suites^{42,44} encompass less than half of the observed variability of Cr# in MORB spinels, and do not include the abundant high-Cr# spinels associated with high-Mg# olivine. We conclude that the abundance of high-Cr# spinel in MORB is a primary, mantle-derived feature, and that the similarity between spinel compositions in Oman dunites and in MORB is not a coincidence.

The Wadi Tayin dunite and harzburgite suites show an inverse correlation between Ti in spinel and Mg# in olivine. Such varia-

tion is consistent with crystallization or dissolution of solid phases with higher Mg# and lower Ti than coexisting liquid (that is, olivine, pyroxenes and/or spinel). However, the small range of variation in olivine Mg#, combined with the large changes in Cr# and Ti concentration in spinel and Ce/Yb in cpx, rules out closed-system crystal fractionation or melting processes. Instead, the magmas which formed these rocks must have undergone open-system processes such as melt/rock reaction⁴⁸ and/or magma mixing⁴⁹. These processes can produce large variations in Cr/Al, Ti and Ce/Yb at nearly constant Mg#. The overlap in olivine Mg#s between dunites and harzburgites indicates that the dunites cannot have formed by partial melting of the harzburgites, or by extensive crystal fractionation of melts derived from the harzburgites. Thus, our geochemical data support the hypothesis that the dunites formed by reaction between ascending melts and mantle peridotite.

Relation to previous studies

We have shown that the Oman dunites formed in upwelling, asthenospheric mantle beneath an oceanic spreading ridge. By contrast, previous studies of dunite geochemistry (for example, refs 21–26, 31, 32, 50, J. E. Quick, personal communication and

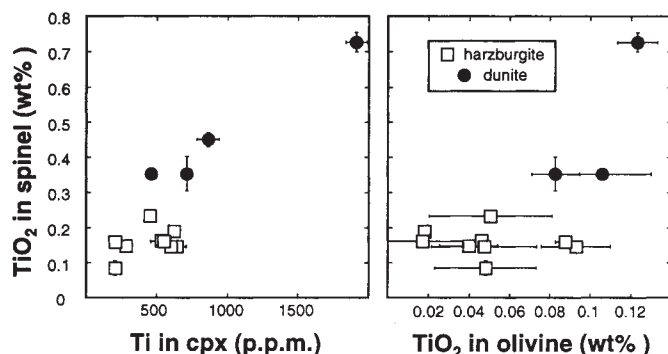


FIG. 5 Variation of TiO_2 concentration in spinel as a function of Ti concentration in cpx and TiO_2 concentration in olivine for dunite and harzburgite samples from the Wadi Tayin mantle section of the Oman ophiolite. Error bars represent one standard deviation from the mean. Correlated Ti contents in different minerals, and higher Ti contents in dunites than in harzburgites, indicate that dunites and harzburgites were equilibrated with distinctly different liquids. (The concentration of TiO_2 in spinel and olivine was determined by electron probe measurements, and the concentration of Ti in cpx by the ion-probe technique.)

E. Takazawa, personal communication, and our unpublished data) have focused on dunite-cutting plagioclase lherzolite in the Horoman, Lanzo, Ronda and Trinity massifs, and on late dunites in the polygenetic Josephine peridotite. Probably, all of these previously studied dunites formed where rising magma migrated into conductively cooled mantle lithosphere. Many were formed in peridotite whose Nd isotopes indicate a long residence time in the lithosphere (for example, Ronda⁵¹, Lanzo²⁵, and Horoman (E. Takazawa, personal communication)). Many are coeval with pyroxenite and gabbro dykes which form on a conductive geotherm. Most cut and replace plagioclase lherzolite, whereas residual plagioclase is not present in the mantle on a normal oceanic adiabat (for example, ref. 3). Plagioclase in abyssal peridotites is not uncommon, but generally results from partial crys-

tallization of migrating liquids where shallow mantle lithosphere is conductively cooled, as at very slow spreading ridges^{40,41}. Available REE and isotopic data for cpx in these previously studied dunites are indicative of equilibrium with liquids significantly different from MORB. We attribute these differences to different parental liquids and different conditions of formation in different tectonic environments.

Mineral compositions similar to those in Oman dunites have been found in the shallow mantle beneath crust formed at the East Pacific Rise, in samples from Hess Deep⁵². Gabbro segregations within dunite have cpx close to REE equilibrium with MORB, whereas cpx in harzburgite surrounding dunite + gabbro is strongly depleted in light REE. Also, spinel in Hess Deep dunite has Cr#s and TiO_2 contents similar to spinel in MORB,

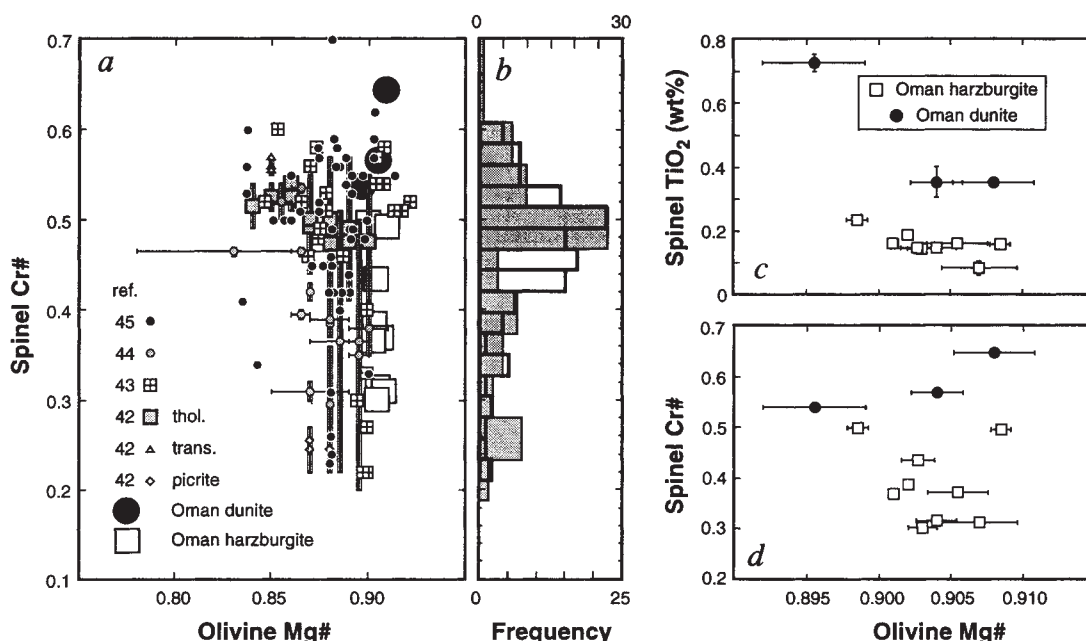


FIG. 6 Variation of spinel Cr# and TiO_2 content as a function of associated olivine Mg# in lavas from mid-ocean ridges and in mantle samples from Oman. All data measured by electron microprobe. They indicate that (1) the abundance of high-Cr# spinels in MORB is a mantle-derived feature, (2) spinel and olivine in Oman dunites are similar to coexisting spinel and olivine in MORB, (3) covariation of spinel and olivine composition in Oman samples is due to open-system processes such as melt/rock reaction or magma mixing, and (4) dunites were not formed by partial melting of harzburgites, but instead formed by reaction between ascending melt and harzburgites. a, Spinel Cr# as a function of associated olivine Mg# in lavas from mid-ocean ridges (refs 42–45) and in Oman mantle samples. Where a range of values for Cr# is reported, this is shown as a vertical, shaded bar with a symbol indicating the

average value. Sigurdsson and Schilling⁴² report a range of Cr# in spinel enclosed in (or attached to) olivine of a given Mg#, in three distinct suites of lavas: tholeiites (data shown as 'thol.'), transitional alkali basalts ('trans.') and picrites. Allan *et al.*⁴⁴ report the range of Cr# and Mg# in individual samples of MORB. Dick and Bullen⁴³ report the compositions of olivine and enclosed or attached spinel in MORB samples. Arai⁴⁵ compiled data on associated spinel and olivine from a variety of sources. b, Histogram of observed Cr# in MORB spinels. Open rectangles indicate frequency for the data compiled by Dick and Bullen⁴³, on a scale of 0 to 25. Shaded rectangles indicate frequency for data compiled in this study, for spinels associated with olivine with Mg# ≥ 0.88 , on a scale of 0 to 30. c and d, detailed spinel and olivine compositional variation in Oman mantle samples. Error bars represent one standard deviation from the mean.

whereas spinel in harzburgite has much lower TiO_2 contents. These similarities support the hypothesis that dunites commonly form in conduits for transport of MORB through the depleted upper mantle. However, the Hess Deep dunites occur in reaction zones around (lithospheric) gabbro dykes, unlike the transposed (asthenospheric) dunites in Oman.

MORB extraction from the asthenosphere

The geochemical data for cpx, spinel and olivine strongly support the hypothesis that the Wadi Tayin dunites formed in equilibrium with liquids similar to MORB, whereas the harzburgites did not. Furthermore, the geochemical data reinforce the conclusion that the dunites formed by replacement of the harzburgites as a result of melt/rock reaction involving dissolution of pyroxene in ascending melt. Finally, field relationships indicate that the dunites formed in the upwelling mantle.

Oman dunites fulfil geochemical, physical and structural requirements for conduits of focused flow in which incremental melts coalesce, mix to form MORB, and are transported to the crust. Ascending melts in dunite conduits would remain far from equilibrium with pyroxene in the shallow mantle. The spatial association of dunite and chromitite, and the geochemical similarity of cpx from both rock types, further reinforces the hypothesis that melt flow is focused in dunites. Because the solubility of Cr-spinel is quite low in silicate melts, chromitites must have scavenged Cr from 300 to 400 times their mass of liquid⁵³. Because dunites completely surround chromitite, the integrated melt/rock ratio for both chromitites and some of the surrounding dunites must have been greater than 300.

Studies employing a thermodynamic approach suggest that dunites form continuously in the adiabatically upwelling mantle

throughout the region of melt migration^{12,16}. If so, dunites must increase in abundance upwards and laterally toward the ridge axis in the upwelling mantle. They then become transposed into a subhorizontal orientation in the shallow mantle, as shown in Fig. 1. Thus, the volume proportion of dunite observed in the shallow mantle section in Oman (5–15%)¹⁸ may represent the total volume of dunite that formed in the entire region of melt migration. The Oman mantle section is 5–15 km thick^{17,18,34}, about one-tenth of the thickness of the melting region^{1–3,6–10}, so the proportion of dunite in the melting region might be 0.5–1.5%. The average degree of partial melting to form MORB is about 5–15% (refs 6–10, 54). If most of these melts pass through dunite conduits, then the average melt/rock ratio in the conduits is about 10.

Regardless of whether the conduits for MORB extraction from the adiabatically upwelling mantle are primarily porous channels, cracks or both, our geochemical data indicate that these conduits are marked by dunites. Geological field relationships, petrologic reasoning and geochemical data indicate that most dunites form as a result of pyroxene-dissolution in magma migrating by porous flow. Although some small dunites in Oman and elsewhere formed in porous reaction zones around magma-filled fractures in the lithosphere, the composition and field relationships of transposed dunites in the mantle section of the Oman ophiolite are entirely consistent with an origin by focusing of porous flow in the asthenosphere. Thus, porous flow plays an important role in the formation of melt conduits and in melt transport within these conduits, suggesting that fracture mechanisms are not essential for extraction of MORB from the asthenospheric mantle beneath mid-ocean ridges. □

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- Allégre, C. J., Montigny, R. & Bottinga, Y. *Bull. Soc. géol. Fr.* **15**, 461–477 (1973).
- Bottinga, Y. & Allégre, C. J. *Tectonophysics* **18**, 1–17 (1973).
- McKenzie, D. & Bickle, M. J. *J. Petrology* **29**, 625–679 (1988).
- O'Hara, M. J. & Scott, J. *Geol.* **1**, 19–40 (1965).
- Stolper, E. *Contr. Miner. Petrol.* **74**, 13–27 (1980).
- Johnson, K. T. M., Dick, H. J. B. & Shimizu, N. *J. geophys. Res.* **95**, 2661–2678 (1990).
- Johnson, K. T. M. & Dick, H. J. B. *J. geophys. Res.* **97**, 9219–9241 (1992).
- Klein, E. & Langmuir, C. H. *J. geophys. Res.* **92**, 8089–8115 (1987).
- Salter, V. J. M. & Hart, S. R. *Nature* **342**, 420–422 (1989).
- Iwamori, H. *Nature* **366**, 734–737 (1993).
- Kelemen, P. B. *J. Petrology* **31**, 51–98 (1990).
- Kelemen, P. B., Whitehead, J. A., Aharonov, E. & Jordahl, K. J. *geophys. Res.* **100**, 475–496 (1995).
- Spiegelman, M. & Kenyon, P. *Earth planet. Sci. Lett.* **109**, 611–620 (1992).
- Hart, S. R. *Proc. natn. Acad. Sci. U.S.A.* **90**, 11914–11918 (1993).
- Nicolas, A. *J. Petrology* **27**, 999–1022 (1986).
- Aharonov, E., Whitehead, J. A., Kelemen, P. B. & Spiegelman, M. *J. geophys. Res.* (in the press).
- Boudier, F. & Coleman, R. G. *J. geophys. Res.* **86**, 2573–2592 (1981).
- Lippard, S. J., Shelton, A. W. & Gass, I. G. *The Ophiolite of Northern Oman* (Blackwell, Oxford, 1986).
- Jackson, M. D. & Ohnenstetter, M. *J. Geol.* **89**, 703–719 (1981).
- Nicolas, A. & Jackson, M. *J. Petrology* **23**, 568–582 (1982).
- Takahashi, N. *Nature* **359**, 52–55 (1992).
- Dick, H. J. B. *Bull. Oregon St. Dep. Geol. miner. Ind.* **96**, 63–78 (1977).
- Quick, J. E. *Contr. Miner. Petrol.* **78**, 413–422 (1981).
- Berger, E. T. & Vannier, M. *Bull. Miner.* **107**, 649–663 (1984).
- Bodinier, J. L., Menzies, M. A. & Thirlwall, M. F. *J. Petrology* (spec. Iherzolites iss.) **191**–210 (1991).
- Kelemen, P. B., Dick, H. J. B. & Quick, J. E. *Nature* **358**, 635–641 (1992).
- Yoder, H. S. *Generation of Basaltic Magmas* (Nat. Acad. Sci., Washington DC, 1976).
- Toramaru, A. & Fujii, N. *J. geophys. Res.* **91**, 9239–9252 (1986).
- von Bagen, N. & Waff, H. S. *J. geophys. Res.* **93**, 1153–1158 (1988).
- Chadam, J., Hoff, D., Merino, E., Ortoleva, P. & Sen, A. *J. appl. Math.* **36**, 207–221 (1986).
- Kelemen, P. B. & Dick, H. J. B. *J. geophys. Res.* **100**, 423–438 (1995).
- Takazawa, E., Frey, F. A., Shimizu, N., Obata, M. & Bodinier, J. L. *Nature* **359**, 55–58 (1992).
- Ceuleneer, G. *Eos* **73**, 537 (1992).
- Nicolas, A. *Structures of Ophiolites and Dynamics of Oceanic Lithosphere* (Kluwer, Dordrecht, 1989).
- Pearce, J. A., Alabaster, T., Shelton, A. W. & Searle, M. P. *Phil. Trans. R. Soc. A300*, 299–317 (1981).
- Shimizu, N. & LeRoex, A. P. *J. Volcan. geotherm. Res.* **29**, 159–188 (1986).
- Hart, S. R. & Dunn, T. *Contr. Miner. Petrol.* **113**, 1–8 (1993).
- Navon, O. & Stolper, E. *J. Geol.* **95**, 285–307 (1987).
- Bodinier, J. L., Vasseur, G., Vernières, J., Dupuy, C. & Fabries, J. *J. Petrol.* **31**, 597–628 (1990).
- Dick, H. J. B. 71–105 (Spec. Publ. 42, Geol. Soc., 1989).
- Elthon, D. J. *geophys. Res.* **97**, 9015–9025 (1992).
- Sigurdsson, H. & Schilling, J. C. *Earth planet. Sci. Lett.* **29**, 7–20 (1976).
- Dick, H. J. B. & Bullen, T. *Contr. Miner. Petrol.* **86**, 54–76 (1984).
- Allan, J. F., Sack, R. O. & Batiza, R. *Am. Miner.* **73**, 741–753 (1988).
- Arai, S. *J. Volcan. geotherm. Res.* **59**, 279–293 (1994).
- Pallister, J. S. & Hopson, C. A. *J. geophys. Res.* **86**, 2593–2644 (1981).
- Evans, B. W. & Frost, B. R. *Geochim. cosmochim. Acta* **39**, 959–972 (1975).
- Kelemen, P. B. *J. Geol.* **94**, 829–843 (1986).
- O'Hara, M. J. & Mathews, R. E. *J. geol. Soc. Lond.* **138**, 237–277 (1981).
- Remaldi, M. thesis, Univ. Montpellier (1993).
- Reisberg, L. & Zindler, A. *Earth planet. Sci. Lett.* **81**, 29–45 (1987).
- Dick, H. J. B. & Natland, J. H. *Proc. ODP Sci. Res.* (in the press).
- Leblanc, M. & Ceuleneer, G. *Lithos* **27**, 231–257 (1992).
- Langmuir, C. H., Klein, E. M. & Plank, T. *Geophys. Monogr.* **71**, 183–280 (1992).
- Anders, E. & Grevesse, N. *Geochim. cosmochim. Acta* **53**, 197–214 (1989).
- Hofmann, A. W. *Earth planet. Sci. Lett.* **90**, 297–314 (1988).
- Sun, S.-S. & McDonough, W. F. *Geol. Soc. Spec. Pub.* **42**, 313–345 (1989).
- Frey, F. A., Walker, N., Stakes, D., Hart, S. R. & Nielsen, R. *Earth planet. Sci. Lett.* **115**, 117–136 (1993).

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Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease

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Some cases of Alzheimer's disease are inherited as an autosomal dominant trait. Genetic linkage studies have mapped a locus (*AD3*) associated with susceptibility to a very aggressive form of Alzheimer's disease to chromosome 14q24.3. We have defined a minimal cosegregating region containing the *AD3* gene, and isolated at least 19 different transcripts encoded within this region. One of these transcripts (*S182*) corresponds to a novel gene whose product is predicted to contain multiple transmembrane domains and resembles an integral membrane protein. Five different missense mutations have been found that cosegregate with early-onset familial Alzheimer's disease. Because these changes occurred in conserved domains of this gene, and are not present in normal controls, they are likely to be causative of *AD3*.

ALZHEIMER'S disease (AD) is a degenerative disorder of the central nervous system which causes progressive memory and cognitive decline during mid to late adult life, and is accompanied by a wide range of neuropathologic features including extracellular amyloid plaques and intra-neuronal neurofibrillary tangles¹. Although the aetiology of this disease is complex², at least three different genetic loci that confer inherited susceptibility to this disease have been identified. The $\epsilon 4$ (112 Cys→Arg) allele of the Apolipoprotein E (*ApoE*) gene is associated with AD in a significant proportion of cases with late onset (>60 years)^{3,4}. Mutations in the gene for the β -amyloid precursor protein (*BAPP*) have been found in a small number of families (<3% of cases) with disease onset before 65 years of age⁵⁻⁸. A third locus (*AD3*) has been mapped by genetic linkage studies to chromosome 14q24.3, and may account for up to 70% of early-onset autosomal dominant AD⁹⁻¹¹. Although early-onset AD is less common than late-onset AD, the *AD3* locus is associated with the most aggressive form of this disease (onset 30 to 60 years),

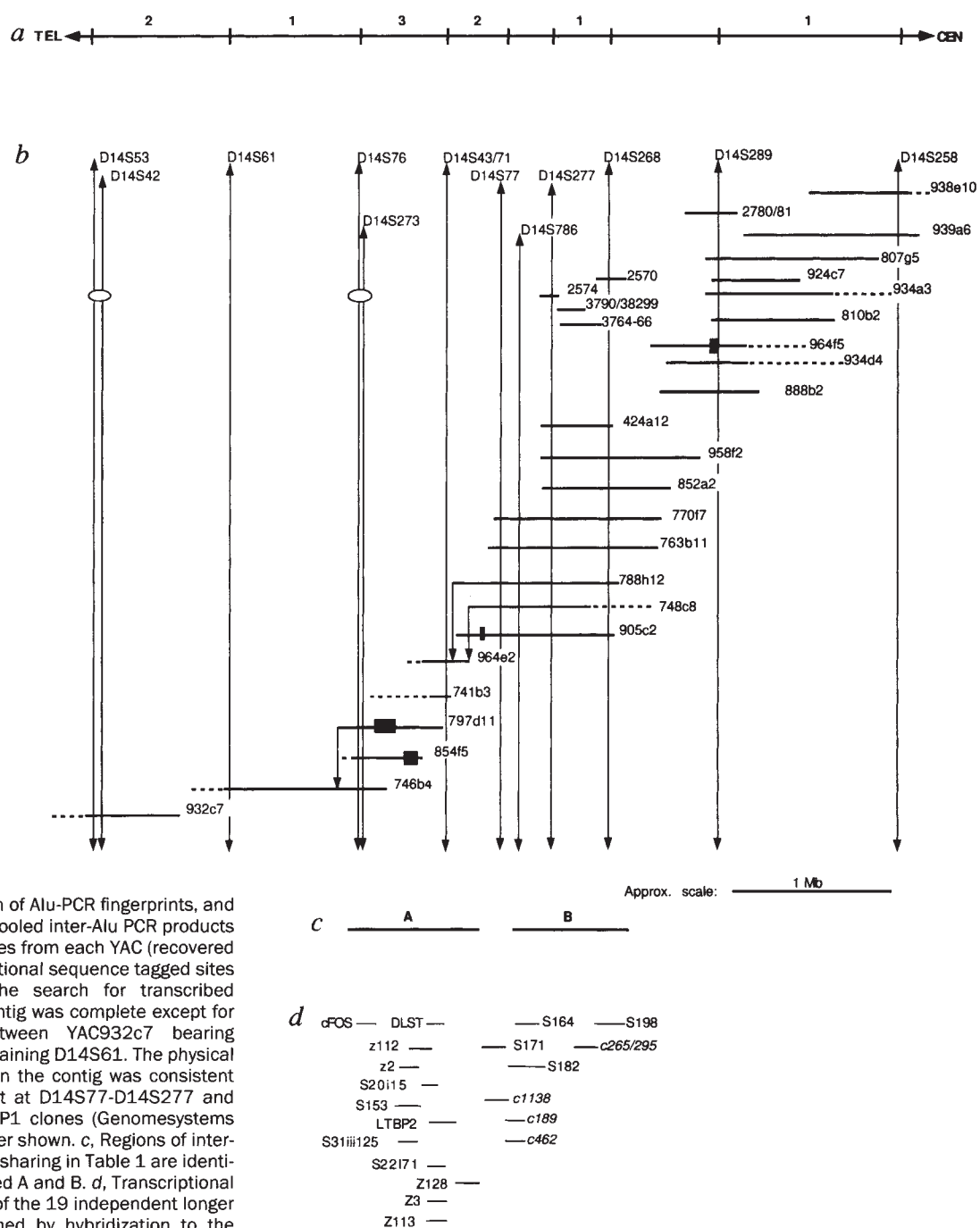
suggesting that mutations at the *AD3* locus put into effect a biologically fundamental process leading to AD. Initial studies of known genes on chromosome 14q resulted in their exclusion as candidates for the *AD3* locus^{9,11-13}. We report the cloning of a novel gene with five missense mutations in seven pedigrees segregating early-onset, autosomal dominant AD.

Genetic mapping of the *AD3* locus

After the initial mapping of the *AD3* locus near the anonymous microsatellite markers D14S43 and D14S53 (refs 9-11), we investigated the segregation of more than 18 additional genetic markers from 14q24.3 (Fig. 1a) in a collection of 21 pedigrees segregating AD as a putative autosomal dominant trait (see ref. 10). Pairwise maximum-likelihood analyses provided substantial cumulative evidence for linkage between familial Alzheimer's disease (FAD) and each of these markers ($z \geq +11.00$). However, many of the genetic data supporting linkage were derived from six large, early-onset pedigrees (FAD1 (ref. 14), FAD2 (ref. 15), FAD3 (refs 16, 17), FAD4 (ref. 18), TOR1.1 (ref. 19) and 603 (ref. 20)), each of which provided an individual log

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FIG. 1 Genetic, physical and transcriptional map of the *AD3* region of chromosome 14. *a*, Genetic map. Inter-marker genetic distances averaged for male and female meioses are indicated in centiMorgans⁴³⁻⁴⁵. The recombination frequencies for the D14S77-D14S277 and D14S268-D14S289 are not provided because the genetic and physical map orders conflict (physical map order is shown). *b*, Physical contig map. YAC and P1 phage clones are represented by horizontal bars drawn proportionally to the length of cloned DNA (horizontal scale bar represents ~1 Mb). Documented chimaerism is depicted by a dotted line; putative interstitial deletions are represented as hatched boxes. The contig was constructed by screening the CEPH MegaYAC human total genomic DNA library with each of the 18 simple sequence repeat (SSR) marker loci used in the genetic linkage studies and with additional unpublished markers (not all tested markers shown)⁴⁶⁻⁴⁸. These clones were arranged into



ordered series by comparison of Alu-PCR fingerprints, and by cross-hybridization with: pooled inter-Alu PCR products from each YAC^{48,49}, end clones from each YAC (recovered by inverse PCR^{3,50}), and additional sequence tagged sites (STSs) generated during the search for transcribed sequences. The resultant contig was complete except for a single discontinuity between YAC932c7 bearing D14S53 and YAC746b4 containing D14S61. The physical map order of the STSs within the contig was consistent with the genetic map except at D14S77-D14S277 and D14S268-D14S289, where P1 clones (Genomesystems Inc., St Louis) predict the order shown. *c*, Regions of interest. The regions of haplotype sharing in Table 1 are identified as horizontal bars labelled A and B. *d*, Transcriptional map. The physical locations of the 19 independent longer cDNA clones were established by hybridization to the panel of *Eco*RI-digested YAC DNA samples. The nucleotide sequence of each of the longer cDNA clones was determined by automated cycle sequencing (Applied Biosystems Inc., California). Accession numbers for partial and complete DNA nucleotide sequences of the transcribed sequences are: L40391, L40392, L40393, L40394, L40395, L40396, L40397, L40398, L40399, L40400, L40401, L40402, L40403, L42110 (human S182) and L42177 (murine S182). Several of the genes within Region A have subsequently been placed upon a syntenic map of *Fugu rubripes* (M. Trower, P. Sanseau and C. W. Dykes, unpublished data).

likelihood ratio (lod score) of greater than +3.00 with at least one marker locus from 14q24.3 (see ref. 10).

Recombinational boundaries defining the location of the *AD3* gene relative to the known locations of the marker loci from 14q24.3 were identified by inspection of the haplotypes of affected members of the six large pedigrees who had been directly genotyped. This minimized the risk of including confounding data arising from unlinked pedigrees, errors in the reconstruction of the genotype of deceased affected subjects, and errors in the estimation of penetrance of the disease trait in elderly asymptomatic subjects. These analyses identified two affected patients

with unequivocal evidence of AD who bore obligate recombination events depicting a telomeric boundary for the *AD3* region at D14S53 (in the affected member from the FAD1 pedigree), and a centromeric boundary at D14S258 (in the affected member from the FAD3 pedigree). Published studies from other pedigrees support localization of *AD3* between D14S53 and D15S258 (ref. 21).

Construction of a physical contig

As an initial step toward the cloning of the *AD3* gene from the minimal cosegregating region, we constructed a contig of

TABLE 1 Extended haplotypes between the centromeric and telomeric flanking markers

Locus	NIH2	FAD3	Tor1.1	FAD4	RB	FAD1	MG12	BOW	COOK	603	Tor42	QUE	MEX1	FAD2
D14S63	1	4	7	4		5							9	2
D14S258	6	6	8	7	4	5	5	6	6		7	6	7	6
D14S268	C	C	B	B	C	C	C	C	C	C	C	B	C	C
D14S277	C	C	C	C	C	C	C	C	C	C	A	C	B	B
D14S786	D	D	E	E	F	E	E	D/F	E	E	E	E	F	D
D14S77	Y	Y	K	S		P	P	K	H		C	U	F	A
D14S71	7	7	1	5	7	7		6	7		3	7	2	6
D14S43	A	A	I	I	I	E	D	I	I		C	I	D	C
D14S273	6	6	5	5	5	4	4	4	6		6	6	5	3
D14S76	5	5	5	5	5	6	9	9			9	1	5	5
D14S61	E	E	G	F		I					D		L	F
D14S53	F	F	C	F	F	J	C	F	E		J	D	F	F
Ethnic origin	Ashk	Ashk	Ital	Ital	Ital	Angl	Angl	Angl	Angl	Amer	FrCan	FrCan	Mex	Ger
Mutation	C410Y	C410Y	M146L	M146L	ND	A246E	ND	ND	ND	H163R	H163R	ND	ND	L286V

The extended haplotypes shown are on the parental copy of chromosome 14 segregating with AD3 in 14 early-onset FAD pedigrees. Identical partial haplotypes (boxed) are observed in several pedigrees of similar ethnic origin. In Region A, alleles of identical size are observed at D14S43 (A: size, 193 bp; normal population frequency, 0.01; C: size, 189 bp; frequency, 0.11; D: size, 187 bp; frequency, 0.12; E: size, 185 bp; frequency, 0.26; I: size, 160 bp; frequency, 0.38); D14S273 (3: size, 193 bp; frequency, 0.38; 4: size, 191 bp; frequency, 0.16; 5: size, 189 bp; frequency, 0.34; 6: size, 187 bp; frequency, 0.02) and D14S76 (1: size, 207 bp; frequency, 0.01; 5: size, 179 bp; frequency, 0.38; 6: size, 177 bp; frequency, 0.07; 9: size, 167 bp; frequency, 0.38). In Region B, shared alleles are seen at D14S268 (B: allele size, 126 bp; allele frequency in normal caucasians, 0.04; C: size, 124 bp; frequency, 0.38); D14S277 (A: size, 158 bp; frequency, 0.06; B: size, 156 bp; frequency, 0.16; C: size, 154 bp; frequency, 0.39); and D14S786 (D: size, 111 bp; frequency, 0.25; E: size, 109 bp; frequency, 0.20; F: size, 107 bp; frequency, 0.47). The ethnic origins of each pedigree are abbreviated as: Ashk, Ashkenazi Jewish; Ital, Southern Italian; Angl, Anglo-Saxon-Celt; FrCan, French-Canadian; Jpn, Japanese; Mex, Mexican caucasian; Ger, German; Am, American caucasian. The type of mutation detected is depicted by the amino-acid substitution and putative codon number, or by ND where no mutation has yet been detected pending a more comprehensive survey for other mutations.

overlapping genomic DNA fragments in yeast artificial chromosome (YAC), P1 bacteriophage, and cosmid vectors (Fig. 1b). Fluorescent *in situ* hybridization (FISH) mapping studies using cosmids derived from the YAC clones 932c7 and 964f5 indicated that this interval was at least five megabases in size. To identify a smaller internal subregion, allelic association methods were applied, but failed to detect statistically significant evidence for linkage disequilibrium at any of the markers within the D14S53–D14S258 interval, even when considering only those pedigrees with early-onset forms of FAD. This result was not unexpected given the diverse ethnic origins of our pedigrees. However, direct inspection of the haplotypes observed on disease-bearing chromosomes from early-onset FAD pedigrees revealed two clusters of markers at which identical alleles were observed in at least two pedigrees with common ethnic origins (Table 1). The first of these clusters was located telomeric to D14S77 (D14S43, D14S273 and D14S76) and spanned the ~1-megabase (Mb) physical interval included within the overlapping YAC clones 964e2, 741b3, 797d11 and part of 854f5 (depicted as region A in Fig. 1c). The second cluster of markers was located immediately centromeric to D14S77 (D14S786, D14S277 and D14S268), and spanned the 0.95-Mb physical interval contained in YAC 788h12 (depicted as region B in Fig. 1c). Significantly, each of the shared extended haplotypes were rare in normal caucasian populations (frequency ≤0.05), and allele sharing was not observed at other

groups of markers spanning similar genetic intervals elsewhere on chromosome 14q24.3. Similar observations have facilitated the cloning of the genes for cystic fibrosis and Huntington's disease^{22,23}, and led to the hypothesis that these results might reflect the co-inheritance of a small physical region surrounding the AD3 gene on the original founder chromosome in each ethnic subgroup.

Transcription mapping

To isolate expressed sequences encoded within both critical intervals, we used a direct selection strategy involving immobilized, cloned, human genomic DNA as the hybridization target to recover transcribed sequences from primary complementary DNA pools derived from human brain messenger RNA²⁴. Approximately 900 putative cDNA fragments of size 100 to 600 base pairs (bp) were recovered from regions A and B in Fig. 1c. These fragments were hybridized to Southern blots containing genomic DNAs from each of the overlapping YAC clones and genomic DNAs from humans and other mammals. This identified a subset of 151 clones which showed evidence for evolutionary conservation and/or for a complex structure which suggested that they were derived from spliced mRNA. The clones within this subset were collated on the basis of physical map location, cross-hybridization and nucleotide sequence, and were used to screen conventional human brain cDNA libraries for longer

FIG. 2 Expression of the S182 transcript. The S182 probe and corresponding longer cDNAs were hybridized to northern blots (Clontech, California) containing 2 µg per lane of human poly-A+ mRNA isolated from various regions of human brain (left) and to blots containing mRNA from other tissues (right). Filters were hybridized at 63 °C for 16 h in 7% SDS, 0.5 M NaPO₄, pH 7.2, 1 mM EDTA, and washed at 63 °C in 40 mM NaPO₄, pH 7.2). The blot containing the mRNA from human brain regions was rehybridized using the APP₆₉₅ probe as a control for mRNA loading (centre).

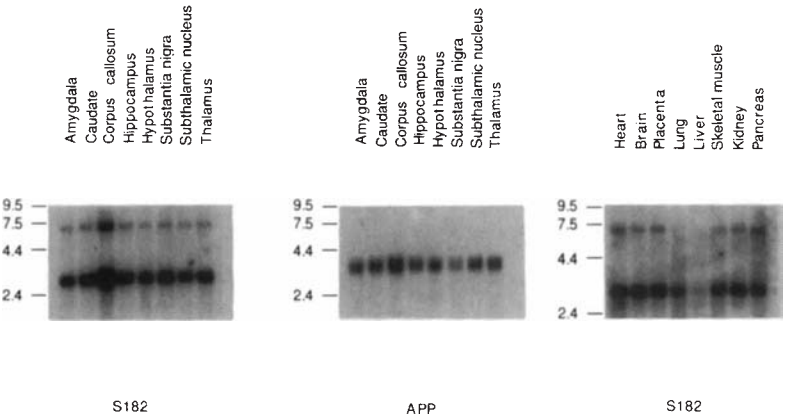




FIG. 3 Putative amino-acid sequences of the human S182 gene (first line), murine S182 homologue (second line), *C. elegans* SPE-4 protein (third line), and human α -1D subunit voltage-dependent Ca^{2+} channel protein (VDCC- α -1D) (fourth line). Identities indicated by asterisk, similarities by vertical line. Amino acids are numbered from the first in-phase ATG codon, which was located 21 codons downstream from a TGA stop codon in the putative 5' UTR (in parentheses) in both species. Transmembrane (TM) domains (underlined) are bounded by residues 82 to 100 (TM-I); 133 to 154 (TM-II); 164 to 183 (TM-III); 195 to 213 (TM-IV); 221 to 238 (TM-V); 244 to 262 (TM-VI); and 408 to 428 (TM-VII). Two smaller hydrophobic domains are located at residues 282 to 299 and 431 to 456, but are not recognized as putative TM membranes by all algorithms. The positions of the mutations are indicated by arrows. Potential phosphorylation sequences exist for MAP kinases (residues 115 and 353) and for protein kinase C (residues 25, 43, 104, 310, 320, 324, 346, 354 and 397). Putative N-glycosylation sequences (indicated by +) exist at Asn 279 and Asn 405. A two-residue shift is made in the third homologous domain of the SPE-4 amino-acid sequence to maximize homology (strong similarities are also present without the two residue shift).

cDNAs. At least 19 independent cDNA clones (>1 kb in length) were isolated and then aligned into a partial transcription map of the *AD3* region (Fig. 1d). Only three of these transcripts corresponded to known characterized genes (cFOS, dihydrolipoamide succinyl transferase and latent transforming growth factor binding protein 2).

Reverse transcriptase polymerase chain reaction (RT-PCR) products corresponding to each transcript were recovered from post-mortem brain tissue or from cultured fibroblast cell lines of affected members of each of the six linked pedigrees, and were screened for sequence differences by comparison with the same products recovered from 12 normal brain tissue samples using chemical cleavage and restriction endonuclease fingerprinting single-strand sequence conformational polymorphism methods^{25,26}. Seven of these transcripts were sequenced because differences were detected between normal and AD-affected subjects, or because a biological role could be postulated from the deduced amino-acid sequence of the candidate gene (for example, S31iii125 has amino-acid homology to Golgi-related glycoproteins that might be involved in β APP processing). These studies led to the discovery of a novel gene, represented by clone S182, which contained a series of nucleotide changes not observed in normal subjects, but which altered the predicted amino-acid sequence in affected subjects. Although nucleotide sequence differences were also observed in some of the other genes, most were in the 3' untranslated regions (UTRs), and none were unique to AD-affected subjects.

Gene characterization

Hybridization of the S182 clone to northern blots identified a major transcript of approximately 3.0 kb and a minor transcript of approximately 7.0 kb, which were expressed in most regions of the human brain and in several peripheral tissues (Fig. 2). Although the identity of the ~7.0 kb transcript is unclear, two observations suggest that the ~3.0 kb transcript represents an active product of the gene. First, and most important, hybridization of the S182 clone to northern blots containing mRNA from a variety of murine tissues, including brain, identifies only a single transcript identical in size to the ~3.0 kb human transcript (not shown). Second, all of the longer cDNA clones recovered so far (size 2.6 to 2.8 kb), which include both 5' and 3' UTRs and which account for the ~3.0 kb band on the northern blot, have mapped exclusively to the same physical region of chromosome 14. From these observations the ~7.0 kb transcript could represent either a rare alternately spliced or polyadenylated isoform of the ~3 kb transcript. The ~7.0 kb signal could also represent another gene with homology to S182. We cannot reject this hypothesis because, although all hybridizing fragments of the S182 open reading frame (ORF) detected in total human genomic DNAs corresponded to those detected in somatic cell hybrids containing only human chromosome 14 (HDM-5 and MHR14; Coriell Institute, Camden, New Jersey), the multiplicity of bands may have obscured fainter signals arising from cross-hybridizing sequences on other chromosomes.

The nucleotide sequence of the human ~3.0 kb major transcript was derived from the consensus of 11 independent longer cDNA clones and from 3 independent clones recovered by standard 5' rapid amplification of cDNA ends. The murine homologue

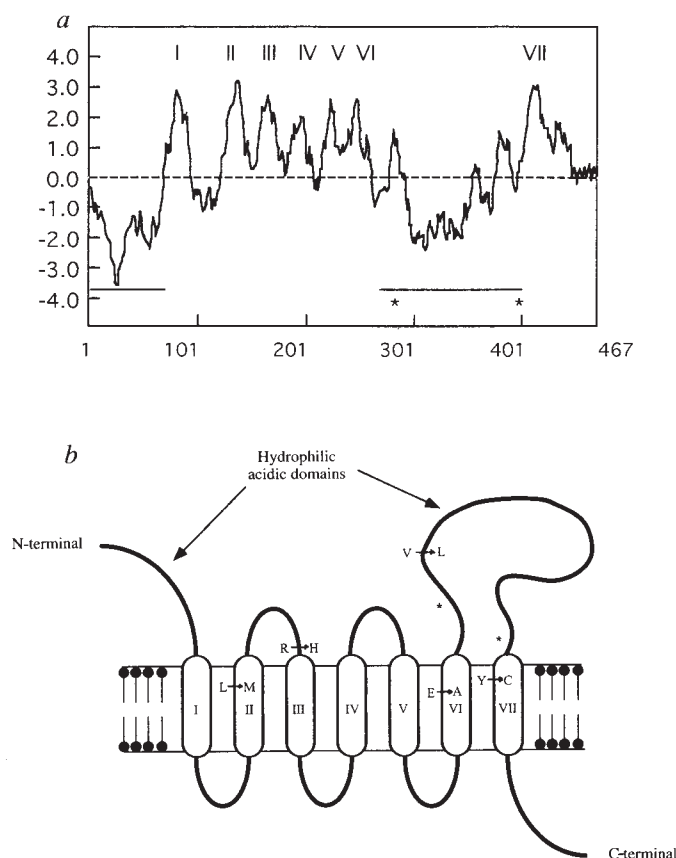


FIG. 4 a, Hydropathy plot of the putative S182 protein using the hydrophobicity indices of Kyte and Doolittle with a calculation window of 15 residues²⁹. Transmembrane domains are numbered I-VII (top), acidic domains are underlined, and N-glycosylation consensus sequences are denoted by an asterisk. b, A model for the structural organization of the putative S182 protein. Roman numerals depict the transmembrane (TM) domains. The internal-external orientation is not known. Putative glycosylation sites (asterisks) and most of the phosphorylation sites (not shown) are located on the same membrane face as the two acidic hydrophilic loops (excepting the MAP kinase site at residue 115 and the protein kinase C site at residue 114). The sites of the FAD mutations are indicated by horizontal arrows.

was derived from the sequence of two partial cDNAs representing the 3' end, and from seven RT-PCR products generated using primers based upon the human 5' sequence. The longest ORF for both the human and murine cDNAs predicts a sequence of 467 amino acids from the first in-phase ATG codon. The amino-acid sequences for both species exhibit 92% identity (Fig. 3). In both species, the first ATG is located 63 bp downstream of a TGA stop codon, and both lack classical Kozak consensus sequences around the first two in-phase ATG sequences²⁷. However, like other genes lacking classical 'strong' start codons²⁷, the putative 5' UTR of both the murine and the human transcripts are rich in GC.

Analysis of the 5' end of the four longest cDNA clones and the RT-PCR products, as well as analysis of corresponding genomic clones, suggest that the 5' UTR is contained within at least two exons and that transcription either begins from two different start sites and/or that one of the early 5' untranslated exons is alternately spliced (E.I.R. *et al.*, unpublished data).

Theoretical predictions on the structure of the protein encoded in the longest ORF (EMBL ProteinPredict and standard hydrophobicity algorithms^{28,29}) suggest that the S182 protein contains at least seven hydrophobic putative transmembrane helical domains linked by hydrophilic sequences, and imply that the S182 protein is an integral membrane protein (Figs 3 and 4). In

addition to the seven classical transmembrane domains, there are also at least two other series of hydrophobic residues, which are shorter in length and would not be predicted to form classical transmembrane domains, but which may be associated with membranes. Assuming then a model with seven hydrophobic transmembrane domains, the acidic amino terminus (residues 1 to 81) and the large acidic linking domain at residues 262 to 408, which also bears two putative N-glycosylation sites at Asn 279 and Asn 405, and which may form a helix bundle, would be positioned on the same membrane face. The apolar carboxy-terminal segment, which may also be membrane associated, would then be located on the opposing membrane face (Fig. 4).

Comparison of the nucleic acid and predicted amino acid sequences with NCBI databases using the BLASTX alignment models revealed amino-acid similarity with the *Caenorhabditis elegans* sperm integral membrane protein SPE-4 ($P = 1.5 \times 10^{-25}$, 24 to 37% identity over 3 groups of at least 50 residues), and weaker similarities to portions of several other membrane-spanning proteins including mammalian chromogranin A and the α -subunit of mammalian voltage-dependent calcium channel ($P = 0.96$, 25 to 47% identity over two groups of at least 19 residues)³⁰ (Fig. 3). The amino-acid sequence similarities across putative transmembrane domains may occasionally yield alignment that simply arises from the limited number of hydrophobic amino acids, but there is also extended sequence alignment between S182 and SPE-4 at several hydrophilic domains (for example at the hydrophilic residues 379 to 402, and 432 to 447 flanking the final predicted transmembrane domain)³¹. Both the putative S182 protein and SPE-4 are predicted to be of comparable size (467 and 465 residues, respectively) and to contain at least seven transmembrane domains with a large acidic domain preceding the final predicted transmembrane domain. The most noticeable structural difference is the longer hydrophilic region at the N terminus predicted for the S182 protein.

Mutations in the S182 gene

Analysis of the nucleotide sequence of the ~3.0 kb S182 transcript recovered by RT-PCR revealed heterozygous nucleotide substitutions in the RT-PCR products from affected members of each of the six large pedigrees (data not shown). Each of these nucleotide substitutions occurred within the putative ORF of the S182 transcript, and would be predicted to change the encoded amino acid at the following positions (numbering from the first putative initiation codon) Met→Leu at codon 146 in pedigrees FAD4 and Tor1.1; His→Arg at codon 163 in pedigree 603; Ala→Glu at codon 246 in pedigree FAD1; Leu→Val at codon 286 in pedigree FAD2; and Cys→Tyr at codon 410 in pedigrees FAD3 and NIH2 (Table 1). The 163 His→Arg mutation found in pedigree 603 was also detected by genomic DNA analysis in a small, unrelated French-Canadian pedigree (Tor42). Like the other pedigrees, Tor42 had an early age of onset (~45 years), but generated only weakly positive lod scores with several genetic markers in the AD3 region ($z \geq +1.00$) because of its small size. Although the 146 Met→Leu, 246 Ala→Glu, 286 Leu→Val and 410 Cys→Tyr mutations were not detected in the remaining smaller pedigrees in our data set, a comprehensive survey for other mutations in these smaller pedigrees must wait until we know the genomic structure of the S182 gene. However, we suspect that mutations in the S182 gene will be very rare in late-onset FAD pedigrees because previous genetic linkage studies have excluded most late-onset FAD pedigrees from chromosome 14 (refs 10, 32).

Of all the nucleotide substitutions cosegregated with the disease in their respective pedigrees (Fig. 5a-e), none were seen in asymptomatic family members aged more than two standard deviations beyond the mean age of onset, and none were present on 284 chromosomes from unrelated neurologically normal subjects drawn from comparable ethnic origins. More importantly, the residues Met 146, His 163, Ala 246, Leu 286 and Cys 410,

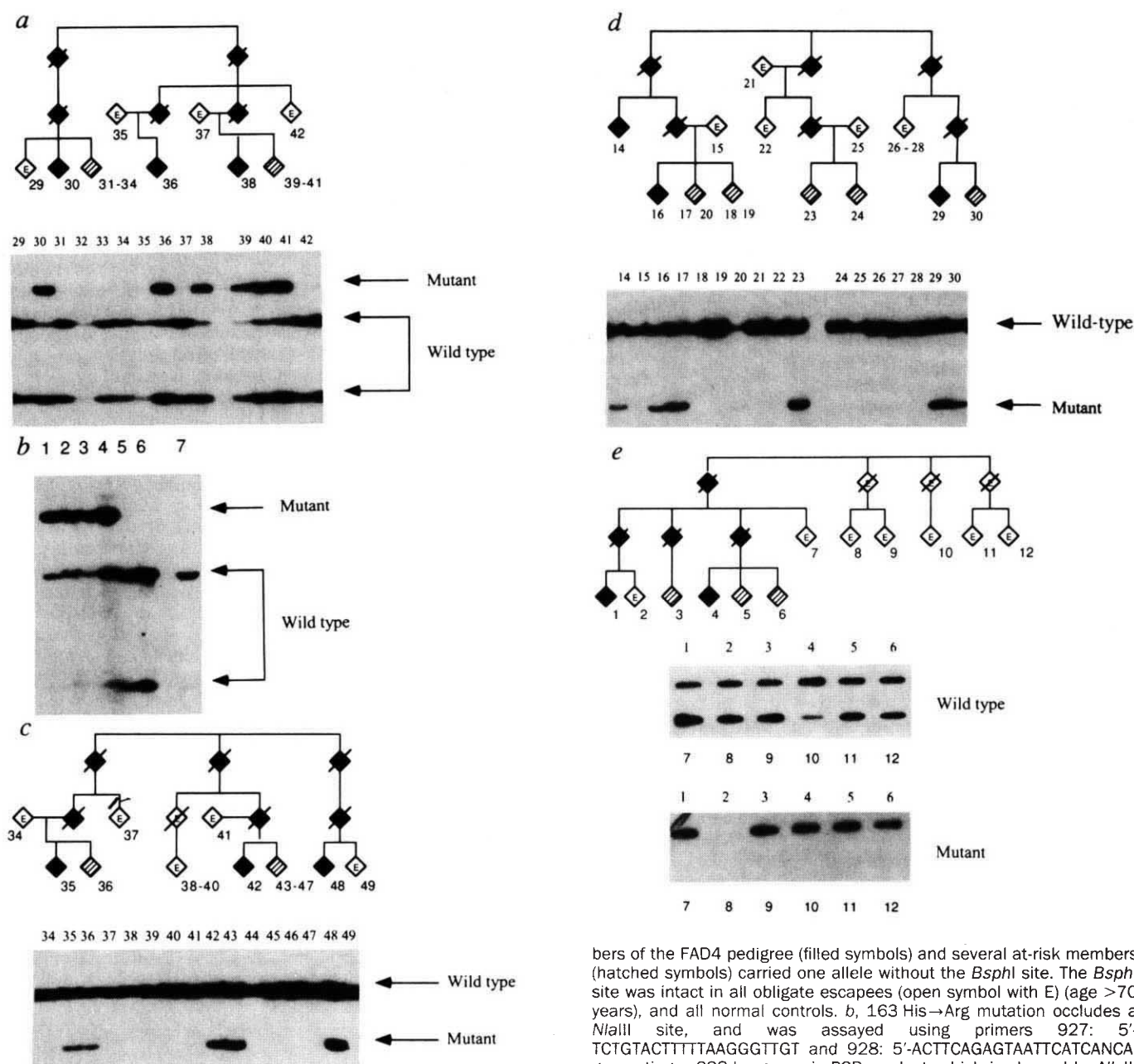


FIG. 5 Analysis of nucleotide substitutions in the S182 gene. Both strands of RT-PCR products (for example, primer pairs 917: 5'-TCA-CATCGGAACAAAACAG and 935: 5'-GAGTCACAGGGACAAAGAGC; 933: 5'-CACCTGAGCAACTACTGTACG and 912: 5'-TCATCTTGCTCCACCACCTG; 901: 5'-AGNAAGATGAGGAAGAAGATG and 1111R: 5'-CACAC-CATTGTTGAGGAGT; 1017F: 5'-GATTTAGTGGCTGTTTGTG and 852: 5'-ACCTCGTCCCTCAAATCT) were directly sequenced by automated *Taq* DNA polymerase cycle sequencing using fluorescent labelled dideoxy-nucleotides (ABI, Foster City, California). Heterozygous nucleotide substitutions were identified from the fluorescence chromatograms by direct inspection and by the Factura (version 1.2.0) and Sequence Navigator (version 1.0.1b15) software packages (data not shown). To confirm the nucleotide sequence differences, and to assess both segregation in FAD pedigrees and frequency in the general population (age >65 years), we devised assays to expedite screening for mutations in the relevant exons recovered from genomic DNA by PCR. a, 146 Met→Leu mutation. The 190-bp PCR product amplified with oligo-nucleotide primers 910: 5'-TCACAGAAGATACCGAGACT and 911: 5'-CCCAACCATAGAAGAAGACAG (reaction conditions: 100 ng genomic DNA, 1.5 mM MgCl₂, 10 pmol of each primer, 0.5 U *Taq* polymerase, 250 μM dNTPs, 1 μCi dCTP in 10 μl reaction volume for 35 cycles of 94 °C for 20 s, 58 °C for 20 s, 72 °C for 5 s) were digested with *Bsp*HI enzyme for 2 h according to the manufacturer's protocols. The resulting restriction fragments (wild type: 107 bp and 83 bp; heterozygous mutation carriers: 107 bp, 83 bp and 190 bp) were resolved on a 6% non-denaturing

bers of the FAD4 pedigree (filled symbols) and several at-risk members (hatched symbols) carried one allele without the *Bsp*HI site. The *Bsp*HI site was intact in all obligate escapees (open symbol with E) (age >70 years), and all normal controls. b, 163 His→Arg mutation occludes a *Nla*III site, and was assayed using primers 927: 5'-TCTGTACTTTTAAAGGGTTGT and 928: 5'-ACTTCAGAGTAATTCATCANCA, generating a 228-bp genomic PCR product, which is cleaved by *Nla*III into 26-bp, 68-bp and 132-bp fragments in the wild type, and into 26-bp and 200-bp fragments owing to loss of the 5' *Nla*III site in the presence of the mutation. Two affected members of the 603 pedigree (lanes 1, 2) and the Tor42 pedigree (lanes 3, 4) pedigrees, as well as normal control subjects (lanes 5 to 7) are shown. c, 246 Ala→Glu mutation creates a *Dde*I site and was assayed using the end-labelled primer 849 (5'-ATCTCCGCGCAGGCATATCT-3') and the unlabelled primer 892 (5'-TGAAATCACAGCCAAGATGAG-3'). The 103-bp wild-type genomic PCR product is not restricted by *Dde*I. The mutation results in restriction into 77-bp and 26-bp fragments. Members of the FAD1 pedigree are shown. d, 286 Leu→Val mutation creates a *Pvu*II site, and was assayed using primers 951: 5'-CACCCATTACAAAGTTTATGC and 952: 5'-GATGAGCAAGTNCCTGAA. The wild-type genomic PCR product does not restrict, and generates a single 262-bp fragment. The mutation results in cleavage of the product into 149-bp and 113-bp fragments. Members of the FAD2 pedigree are shown. e, 410 Cys→Tyr mutation. A 216-bp PCR product was amplified from 100 ng genomic DNA with primers 885: 5'-TGGAGACTGGAACACAAC and 893: 5'-GTGTGGCCAGGGTAGAGAACT, denatured by tenfold dilution in 0.4 M NaOH, 25 mM EDTA, and vacuum slot-blotted to duplicate nylon membranes. The genotype was scored by differential hybridization of 'wild type' primer 890: 5'-CCATAGCCTGTTTCGTAGC and 'mutant' primer 891: 5'-CCATAGCCTATTTTCGTAGC to duplicate filters (hybridization: 5× SSC, 5× Denhardt's, 0.5% SDS for 1 h at 48 °C; washing: 2× SSC, 0.1% SDS at 50 °C). Members of the FAD3 pedigree are shown.

which were altered in the human FAD pedigrees, are conserved in the murine homologue and occur in highly conserved domains of the putative S182 protein (Fig. 4). These results suggest that these nucleotide substitutions are pathogenic mutations rather than innocent polymorphisms.

Discussion

Our results provide strong evidence that mutations in the *S182* gene are the cause of early onset FAD in some pedigrees, but two important questions arise. First, what is the physiologic function of this gene? Second, how do these mutations confer a dominantly inherited AD phenotype?

The general topology of the putative S182 protein suggests that it is an integral membrane protein, such as a receptor, a channel protein, or a structural membrane protein. Although the absence of a recognizable signal peptide and the paucity of glycosylation sites are noteworthy, the hydropathy profile suggests that the protein is less likely to be a soluble protein with a highly compact three-dimensional structure. The expression of S182 in non-neural tissues does not necessarily negate its role in AD because the genesis of the AD phenotype probably requires interactions with other gene products³³.

The similarity between the putative products of the *spe-4* and *S182* genes implies that they may have similar activities. The SPE-4 protein of *C. elegans* appears to be involved in the formation and stabilization of the fibrous body-membrane organelle (FBMO) complex during spermatogenesis. The FBMO is a specialized Golgi-derived organelle, consisting of a membrane-bound vesicle attached to and partly surrounding a complex of parallel protein fibres (predominantly formed from the major sperm protein), and is probably involved in the transport and storage of soluble and membrane-bound polypeptides³¹. Mutations in *spe-4* disrupt the FBMO complexes and arrest spermatogenesis. The physiologic function of SPE-4 therefore may be either to stabilize interactions between integral membrane proteins within the FBMO membrane during membrane budding and fusion events, or to stabilize interactions between the membrane and fibrillary proteins (including the parallel major sperm protein fibrils) during the intracellular transport of the FBMO complex during spermatogenesis.

Comparable functions could be envisaged for the putative S182 protein. It could be involved either in the docking of other membrane-bound proteins such as β APP, or in the axonal transport and fusion budding of membrane-bound vesicles during

protein transport (for example, in the Golgi apparatus, in the endosome-lysosome system, or in synapses).

If this speculation is correct then the mutations we have found in FAD kindreds might be expected to result, for example, in aberrant transport and processing of β APP, and/or abnormal interactions with cytoskeletal proteins such as the microtubule-associated protein Tau. Abnormalities in the intracellular, and ultimately in the extracellular, disposition of both of these proteins are an integral part of the neuropathologic features of AD^{34,35}. Although the location of the S182 mutations in conserved domains of the putative protein suggests that they are pathogenic, at least three of these mutations are conservative (which is commensurate with the onset of disease in adult life). Because none of the mutations observed so far are deletions or nonsense mutations that would be expected to cause a loss of function, we cannot predict whether these mutations will have a dominant gain-of-function effect (perhaps promoting aberrant processing of β APP) or a dominant loss-of-function effect (perhaps causing arrest of normal β APP processing).

An alternative possibility is that the *S182* gene product may represent a receptor or channel protein. Mutations of such proteins have been causally related to several other dominant neurological disorders in both vertebrate (for example malignant hyperthermia and hyperkalemic periodic paralysis in humans^{36,37}) and in invertebrate organisms (for example the *mec-4(d)* and *deg-1(d)* mutants in *C. elegans*^{38,39}). Although the pathology of these other disorders does not resemble that of AD, there is evidence for functional abnormalities in ion channels in AD. For instance, anomalies have been reported in the tetraethylammonium-sensitive 113pS potassium channel and in Ca^{2+} homeostasis⁴⁰⁻⁴². Perturbations in transmembrane Ca^{2+} fluxes might be especially relevant in view of the admittedly quite weak homology between S182 and the α -1D subunit of voltage-dependent calcium channels (VDCC- α -1D), and the observations that increases in intracellular Ca^{2+} in cultured cells can replicate some of the biochemical features of AD (for example, alterations in the phosphorylation of Tau-microtubule-associated protein and increased production of A β peptides)^{41,42}.

Although the exact mechanism by which Alzheimer-type neurodegeneration occurs in the brains of humans heterozygous for these mutations will require further study, and may not in the end relate to any of our hypotheses, it will be important to discern where the *S182* gene product is localized, and whether it serves as a receptor, as an ion channel or has a structural role. It will also be of interest to discover how it associates with β APP or ApoE in the pathogenesis of AD. □

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- Katzman, R. *N. Engl. J. Med.* **314**, 964-973 (1986).
- St George-Hyslop, P. H. et al. *Nature* **347**, 194-197 (1991).
- Strittmatter, W. J. et al. *Proc. natn. Acad. Sci. U.S.A.* **90**, 1977-1981 (1993).
- Saunders, A. et al. *Neurology* **43**, 1467-1472 (1993).
- Goate, A. M. et al. *Nature* **349**, 704-706 (1991).
- Chartier-Harlin, M.-C. et al. *Nature* **353**, 844-846 (1991).
- Murrell, J., Farlow, M., Ghetti, B. & Benson, M. D. *Science* **254**, 97-99 (1991).
- Karlinsky, H. et al. *Neurology* **42**, 1445-1453 (1992).
- Schellenberg, G. D. et al. *Science* **258**, 668-670 (1992).
- St George-Hyslop, P. et al. *Nature Genet.* **2**, 330-334 (1992).
- Van Broeckhoven, C. et al. *Nature Genet.* **2**, 335-339 (1992).
- Rogaev, E. I. et al. *Neurology* **43**, 2275-2279 (1993).
- Wong, L. et al. *Neurosci. Lett.* **152**, 96-98 (1993).
- Nee, L. et al. *Archs Neurol.* **40**, 203-208 (1983).
- Frommelt, P., Schnabel, R., Kuhne, W., Nee, L. E. & Polinsky, R. J. *Alzheimer Dis. Ass. Disorders* **5**, 36-43 (1991).
- Goudsmit, J. et al. *J. Neurol. Sci.* **49**, 78-89 (1981).
- Pollen, D. *Hannah's Heirs: the Quest for the Genetic Origins of Alzheimer's Disease* (Oxford Univ. Press, Oxford, 1993).
- Foncin, J.-F. et al. *Rev. Neurol.* **141**, 194-202 (1985).
- Bergamini, L. et al. *Acta Neurol.* **13**, 534-538 (1991).
- Pericak-Vance, M. A., Yamaoka, L. H. & Haynes, C. S. *Exp Neurol.* **102**, 271-279 (1988).
- Cruts, M. et al. *Neurobiol. Aging* **15**, S129 (1994).
- Kerem, B.-S. et al. *Science* **245**, 1073-1080 (1989).
- Gusella, J. F. & Group, H. D. C. *Cell* **72**, 971-983 (1993).
- Rommens, J. M. et al. *Hum. molec. Genet.* **2**, 901-907 (1993).
- Saleeba, J. A. & Cotton, R. G. *Meth. Enzym.* **217**, 285-295 (1993).
- Liu, Q. & Sommer, S. S. *BioTechniques* **18**, 470-476 (1995).
- Kozak, M. J. *biochem. J.* **266**, 19867-19870 (1991).
- Rost, B. & Sander, C. *Proteins* **19**, 55-72 (1994).
- Kyte, J. & Doolittle, R. F. *J. molec. Biol.* **157**, 105 (1982).
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. *molec. Biol.* **215**, 403-410 (1990).

- L'Hernault, S. W. L. & Arduengo, P. M. *J. Cell Biol.* **119**, 55-69 (1992).
- Schellenberg, G. D. et al. *Am. J. hum. Genet.* **53**, 619-628 (1993).
- St George-Hyslop, P. H. et al. *Science* **263**, 536-537 (1994).
- Kosik, K. & Greenberg, S. M. in *Alzheimer Disease* (eds Terry, R. D., Katzman, R. & Bick, K. L.) 335-344 (Raven, New York, 1994).
- Selkoe, D. J. A. *Rev. Neurosci.* **17**, 489-517 (1994).
- MacLennan, D. H. et al. *Nature* **343**, 559-561 (1990).
- Placek, L. J. et al. *Cell* **67**, 1021-1027 (1991).
- Chalfie, M. & Wolinsky, E. *Nature* **345**, 410-416 (1990).
- Driscoll, M. & Chalfie, M. *Nature* **349**, 588-593 (1991).
- Etcheberrygaray, R. et al. *Proc. natn. Acad. Sci. U.S.A.* **90**, 8209-8213 (1994).
- Mattson, M. P. et al. *Trends Neurosci.* **16**, 409-414 (1993).
- Querfurth, H. W. & Selkoe, D. J. *Biochemistry* **33**, 4550-4561 (1994).
- Weissenbach, J. et al. *Nature* **359**, 794-798 (1992).
- NIH/CEPH Collaborative Mapping Group. *Science* **258**, 67-86 (1992).
- Gyapay, G. et al. *Nature Genet.* **7**, 246-339 (1994).
- Albertsen, H. M. et al. *Proc. natn. Acad. Sci. U.S.A.* **87**, 4256-4260 (1990).
- Chumakov, I. M. et al. *Nature Genet.* **1**, 222-225 (1992).
- Ioannu, P. A. et al. *Nature Genet.* **6**, 84-89 (1994).
- Chumakov, I. et al. *Nature* **359**, 380-387 (1992).
- Riley, J. et al. *Nucleic Acids Res.* **18**, 2887-2890 (1990).

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Primary neurogenesis in *Xenopus* embryos regulated by a homologue of the *Drosophila* neurogenic gene *Delta*

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***X-Delta-1*, a *Xenopus* homologue of the *Drosophila* *Delta* gene, is expressed in the early embryonic nervous system in scattered cells that appear to be the prospective primary neurons. Ectopic *X-Delta-1* activity inhibits production of primary neurons and interference with endogenous *X-Delta-1* activity results in overproduction of primary neurons. These results indicate that the *X-Delta-1* protein mediates lateral inhibition delivered by prospective neurons to adjacent cells, and that commitment to a neural fate in vertebrates is regulated by *Delta*–*Notch* signalling as in *Drosophila*.**

THE vertebrate central nervous system is an intimate mixture of different cell types, almost all generated from the same source—the neurogenic epithelium that forms the neural plate and subsequently the neural tube. What are the mechanisms that control neurogenesis in this sheet of cells, directing some to become neurons while others remain non-neuronal? The answer is virtually unknown for vertebrates, but many of the cellular interactions and genes controlling cell-fate decisions during neurogenesis have been well characterized in *Drosophila*¹. Although the gross anatomical context of neurogenesis appears to be very different in insects and vertebrates, the possibility remains that, at a cellular level, similar events are occurring through conserved molecular mechanisms.

Neural precursors arise in the *Drosophila* embryo from a neurogenic epithelium during successive waves of neurogenesis^{2,3}. The pattern of production of these cells is largely determined by the activity of the proneural and neurogenic genes. Proneural genes predispose clusters of cells to a neural fate (reviewed in ref. 4), but only a subset of cells in a cluster become neural precursors. This restriction is due to the action of the neurogenic genes, which mediate lateral inhibition—a type of inhibitory cell signalling by which a cell committed to a neural fate forces its neighbours either to remain uncommitted or to enter a non-neural pathway^{5,6}. Mutations leading to a failure of lateral inhibition cause an overproduction of neurons—the ‘neurogenic’ phenotype^{7,8}. In *Drosophila*, the inhibitory signal is delivered by a transmembrane protein encoded by the *Delta* neurogenic gene, which is displayed by the nascent neural cells⁹. Neighbouring cells express a transmembrane receptor protein, encoded by the neurogenic gene *Notch*¹⁰. Both proteins span the membrane once and include multiple tandem epidermal growth factor (EGF)-like repeats in their extracellular domains¹¹. Homologues are found in *Caenorhabditis elegans*, where the *Notch*-related gene *lin-12* and the *Delta*-related gene *lag-2* are also responsible for lateral inhibition^{12–14}. In vertebrates, several *Notch* homologues have also been identified^{15–21}, and they are expressed in many tissues and at many stages of development. Loss of *Notch-1* leads to somite defects and embryonic death in mice^{22,23}, whereas constitutively active mutant forms of *Notch-1* appear to inhibit cell differentiation in *Xenopus* and in cultured mammalian cells^{24–26}. However, it has been difficult to pin down

specific roles for *Notch* in vertebrate neural development. *Notch* genes may have multiple overlapping functions, and we lack information about vertebrate *Notch* ligands.

We have isolated a *Xenopus* homologue of the *Drosophila* *Delta* gene, called *X-Delta-1* (ref. 27). Here we examine the function of *X-Delta-1* in the development of the early embryonic nervous system. We show that *X-Delta-1* expression prefigures expression of neuronal markers, indicating that the gene is active in prospective neurons (see also accompanying paper²⁷). We show that neurogenesis is sensitive to *X-Delta-1* activity: raised activity causes fewer cells to become primary neurons, whereas reduced activity causes more cells to do so. In addition, we show that *X-Delta-1* and *X-Notch-1* (formerly called *Xotch*²⁰) are expressed in similar domains of the neural plate, and that the effects of constitutively activating *X-Notch-1* resemble those of over-expressing *X-Delta-1*. Together, these results provide evidence that lateral inhibition operates in vertebrates via *Notch* and *Delta* homologues, regulating the choices that individual cells make so as to produce a fine-grained pattern of neuronal differentiation.

The pattern of primary neurons

The conserved structural features found in candidate ligands for *Notch* family members in both *Drosophila* and *C. elegans*^{13,28,29} allowed us to use the polymerase chain reaction (PCR) to identify a *Xenopus* homologue of *Drosophila* *Delta*, called *X-Delta-1* (ref. 27). *X-Delta-1* and the chick *Delta* homologue *C-Delta-1* code for proteins that share significant sequence homology to *Drosophila* *Delta*, leading us to ask whether the vertebrate genes are also involved in regulating commitment to a neural fate. If *X-Delta-1* is involved in selecting neuronal precursors in the frog neuroepithelium, it would be expressed in regions where neurons are being generated. We therefore analysed the expression of *X-Delta-1* RNA during neurogenesis, focusing on the formation of the early nervous system.

In *Xenopus*, as in other lower vertebrates, an initial wave of neurogenesis gives rise to a simple pattern of so-called primary neurons, whose early, precisely scheduled development and simple layout make primary neurogenesis an attractive model for the study of mechanisms involved in neural cell fate decisions^{30–32}. Primary neurons arise initially in the posterior

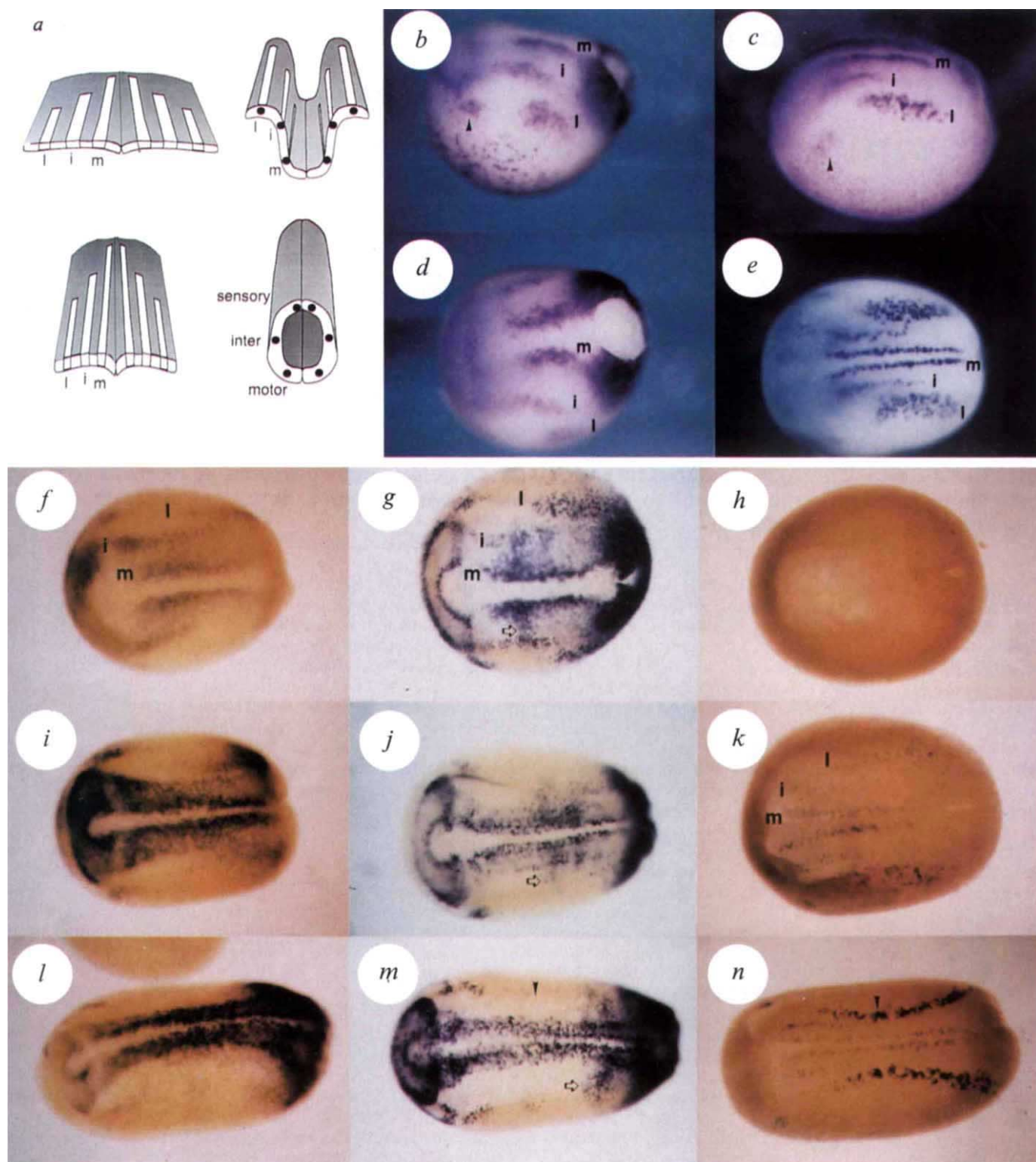


FIG. 1 Early expression of *X-Delta-1* matches the pattern of primary neurogenesis in the *Xenopus* embryo. *a*, Schematic diagram of the pattern of primary neurogenesis in the *Xenopus* embryo. Primary neuronal precursors arise in three stripes on either side of the midline of the neural plate, marked in this and subsequent figures: m (medial), i (intermediate) and l (lateral). As the plate elongates and narrows, these domains shift and eventually come to occupy ventral, intermediate and dorsal positions in the neural tube, where they differentiate as motor neurons, interneurons and sensory neurons, respectively. *b–e*, *In situ* hybridization showing expression of *X-Delta-1* (*b*, *d*) or *N-tubulin* (*c*, *e*) in uncleared specimens, with anterior to the left (as in subsequent figures). *b*, *X-Delta-1* at stage 12, side view; *c*, *N-tubulin* at stage 13.5, side view;

d, *X-Delta-1* at stage 12, dorsal view; *e*, *N-tubulin* stage 14, dorsal view. Early expression of *X-Delta-1* in *b* and *d* prefigures the expression of *N-tubulin* in *c* and *e*; the stripes have moved because of convergent-extension movements. Staining in the trigeminal ganglion is indicated with an arrowhead. *f–n*, *In situ* hybridization showing that *X-Notch-1* and *X-Delta-1* are coexpressed and prefigure *N-tubulin* expression; cleared embryos during neural plate stages, viewed from the dorsal side. *f–h*, stage 12; *i–k*, stage 14; *l–n*, stage 17. Left column (*f*, *i*, *l*) *X-Notch-1*; middle column (*g*, *j*, *m*) *X-Delta-1*; right column (*h*, *k*, *n*) *N-tubulin*. Expression of *X-Notch-1* overlaps extensively with that of *X-Delta-1* and also occurs in three stripes. Both are present before *N-tubulin* has begun to be expressed (compare *f* and *g* with *h*). By stage 17, when

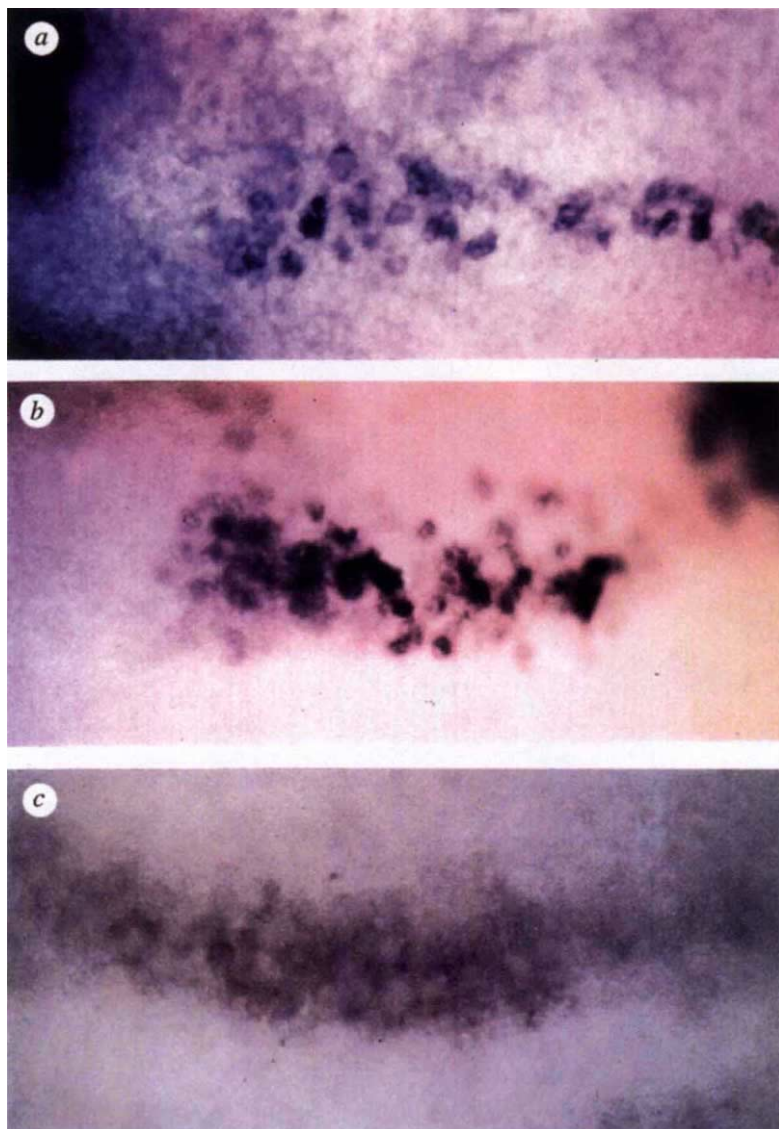


FIG. 2 *X-Delta-1* and *N-tubulin* are expressed in scattered cells in the medial neurogenic stripe of the neural plate; *X-Notch-1* is expressed more uniformly in the same domain. Dorsal views of whole-mount *in situ* hybridization specimens, with anterior to the left. a, *X-Delta-1*, stage 12; b, *N-tubulin*, stage 13; c, *X-Notch-1*, stage 12; a shows the left side of the embryo, b and c show the right side.

part of the neural plate. Their genesis can be followed by monitoring the expression of a neuron-specific type-II β -tubulin gene, hereafter referred to as *N-tubulin*, which is a very early differentiation marker for the primary neurons^{33,34} (data not shown). *N-tubulin* expression is detected by *in situ* hybridization in the neural plate beginning at stage 12.5–13.0, in scattered cells that are organized in three longitudinal domains—medial, intermediate, and lateral—on either side of the midline (Fig. 1a, c, e). As

the neural plate folds to form the neural tube, the medial patch comes to lie ventrally in the tube, where the cells differentiate as a column of motor neurons; the cells of the intermediate patch differentiate as a column of interneurons; the most lateral patch of cells end up most dorsally in the tube, where they differentiate as primary sensory neurons (Rohon-Beard cells) (Fig. 1a).

By whole-mount *in situ* hybridization, *X-Delta-1* expression in the nervous system is first seen at the end of gastrulation (stage 12), in scattered cells in specific domains of the neural plate (Fig. 1b, d), around the period when the primary neuronal precursors are undergoing their final round of DNA replication, and about two hours before cells show the first signs of neuronal differentiation³⁰. As shown in Fig. 1, the initial pattern of expression of *X-Delta-1* in the neural plate prefigures exactly, in shape and position, the three longitudinal domains in which primary neuronal precursors arise (compare Fig. 1b to c and d to e). Expression of *X-Delta-1* anticipates that of *N-tubulin* by at least two hours: for example, the three longitudinal stripes of *X-Delta-1* expression arise at stage 12 when the neural plate is extremely broad, but the corresponding stripes of *N-tubulin* expression only appear at stage 12.5–13.0, after the plate has narrowed by convergent extension (Fig. 1; compare b, c; d, e; g, k). Moreover, *X-Delta-1* switches off as *N-tubulin* expression switches on (for example, compare the lateral stripes in Fig. 1m and n). In a similar way, *X-Delta-1* is subsequently expressed in regions of (secondary) neurogenesis but not in mature neurons (data not shown²⁷). In sum, these results suggest that *X-Delta-1* is expressed in prospective neurons at around the time of their commitment to a neuronal fate.

Expression of *X-Delta-1* and *X-Notch-1*

If *X-Delta-1* encodes a ligand for the X-Notch-1 protein, domains of *X-Delta-1* and *X-Notch-1* expression should overlap. *In situ* hybridization shows that this is indeed the case: *X-Notch-1* is also expressed in three longitudinal stripes in the posterior neural plate, overlapping with *X-Delta-1* expression and likewise anticipating *N-tubulin* expression (Fig. 1f). Whereas *X-Delta-1* and *N-tubulin* are expressed in scattered, isolated cells, *X-Notch-1* expression appears diffuse (Fig. 2), as though present in most or all of the cells in the local population. This is the pattern expected if *X-Delta-1* and *X-Notch-1* are responsible for restricting neuronal development to isolated cells by lateral inhibition.

Delta-1 and *X-Notch-1* are responsible for restricting neuronal development to isolated cells by lateral inhibition.

Inhibition of primary neurogenesis

By overexpression of *X-Delta-1*. Experiments using *Drosophila* genetic mosaics imply that a cell expressing *Delta* activates Notch in neighbouring cells, inhibiting them from becoming neural precursors⁹. To test whether *X-Delta-1* similarly inhibits the production of neurons in *Xenopus*, we injected a mixture of *in vitro*-transcribed *X-Delta-1* and *lacZ* transcripts into one blastomere of two-cell stage embryos, and analysed *N-tubulin* expression in embryos at the neural plate stage. The *lacZ* serves to indicate the distribution of injected transcripts, and the uninjected side of the embryo serves as a control.

Injection of 0.1–1 ng of *X-Delta-1* RNA leads to a striking reduction or complete elimination of *N-tubulin*-expressing cells on the injected side (Fig. 3a, b, c, e and Table 1), whereas injection of *lacZ* transcripts alone has no such effect (Fig. 3d). The sites where neurons are eliminated correlates with the distribution of injected transcripts, as indicated by X-gal staining for *lacZ* expression. Single injections lead to a regionalized distribu-

▶ *N-tubulin* expression in the lateral stripe is well established (arrowhead in n), expression of *X-Delta-1* is gone (arrowhead in m). Transverse bands of *X-Delta-1* expression in cleared embryos (open arrows in g, j, m) lie in the dorsal (somitic) mesoderm below the neural plate (see also ref. 27), and move posteriorly as somitogenesis proceeds. *Delta-1*'s role in somitogenesis is unknown, although *Notch-1* appears to be required for stable maintenance of somitic boundaries²³.
METHODS. Whole-mount *in situ* hybridization⁴⁵ used digoxigenin-labelled antisense RNA probes, derived from cDNA for *X-Delta-1*, and as previously described for *Notch-1* and *N-tubulin*^{20,33}. Stained embryos were postfixed for two days in MEMFA and photographed directly, or after clearing with benzyl benzoate⁴⁵.

FIG. 3 Primary neurogenesis is suppressed by ectopic expression of *X-Delta-1* or constitutively activated *X-Notch-1*. Test RNAs mixed with marker *lacZ* transcripts were injected into single blastomeres of two-cell-stage embryos, fixed at neural plate stages, and stained for β -galactosidase with X-gal to identify the injected side (sky-blue), and subjected to whole-mount *in situ* hybridization (purplish blue) for expression of *N-tubulin* (a–e, g and h) or *X-Delta-1* (i, j). a–c, Single injections of synthetic *X-Delta-1* RNA transcripts (0.4–1 ng) gave localization of the transcripts in the lateral (a), intermediate (b), or medial (c) neural plate as shown by X-gal staining (brackets). Corresponding stripes of *N-tubulin* expression are lost. The three stripes of *N-tubulin* staining on the uninjected side are marked m (medial), i (intermediate) and l (lateral). d, Control injection of *lacZ* RNA alone; *N-tubulin* expression is unaffected. Similar results were obtained with a form of *X-Delta-1* consisting of only the extracellular domain. e, Section through neural-plate-stage embryo injected several times on one side at the two-cell stage with *X-Delta-1* and *lacZ* RNA. *N-tubulin* expression is lost on the injected side. f, Section through an embryo injected with *X-Delta-1* and *lacZ* RNA, and immunostained for Islet-1 to show differentiated neurons. Cells that express Islet-1 are absent from the injected side (left). The Islet-1 staining cells on the uninjected side (arrowhead) are interneurons. Note that the injected side of the neural tube is abnormally thin-walled, probably due to lack of large primary neurons. g, h, Ectopic expression of activated *Notch* ΔE^{24} suppresses *N-tubulin* expression. g, Dorsal view of an uncleared embryo showing that *N-tubulin* staining is reduced on the injected side (top). *Notch* ICD acts similarly (not shown). h, Section through an embryo processed as in g. Loss of *N-tubulin*-expressing cells in lateral regions of the neural plate corresponds to the site of injected RNA. i, Constitutive *Notch* activity suppresses *X-Delta-1* expression. Embryo was injected with *Notch* ICD and *lacZ* RNAs, and processed for X-gal staining (bracketed; faint in this specimen) and by whole-mount *in situ* hybridization for *X-Delta-1* RNA. *X-Delta-1* staining is lost from the injected region. j, *X-Delta-1* staining is normal in control embryos injected with *lacZ* RNA.

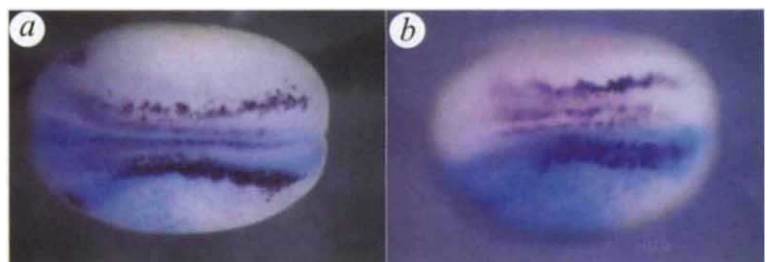
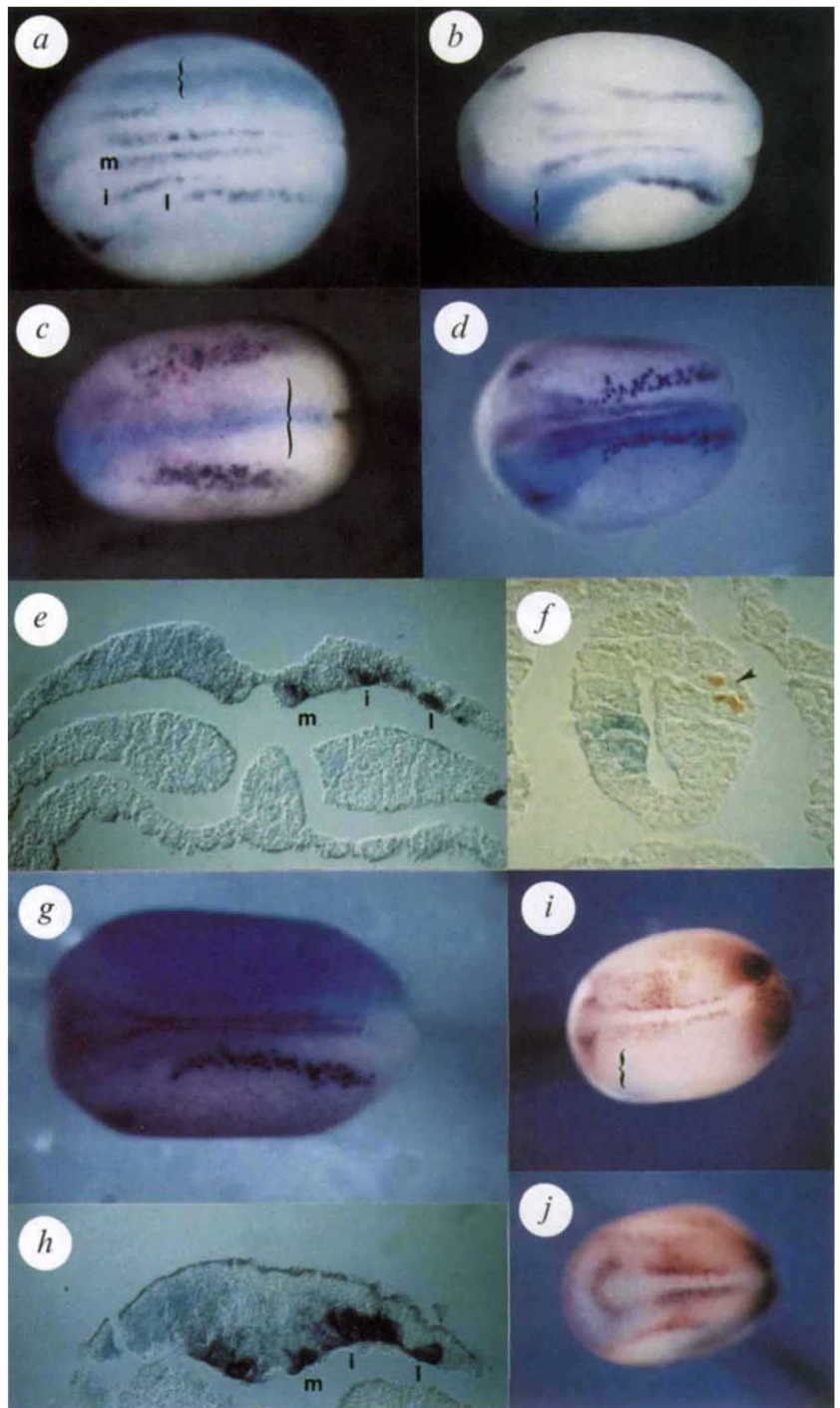
METHODS. Synthetic *lacZ* (20 pg), and *X-Delta-1* (100 pg) or *Notch* ΔE (100 pg) or *Notch* ICD (100 pg) RNAs were injected into single blastomeres of embryos at the two-cell stage, as described previously²⁴. The coding region of *X-Delta-1* was subcloned into the CS2 vector⁴⁶ (from D. Turner), and transcribed into capped RNA using SP6 polymerase²⁴. Unless noted, RNA was injected in a volume of 10 nl, at a concentration of 2–100 ng μ l⁻¹; each RNA sample was injected into a batch of 30–50 embryos. *In situ* hybridization shows that injected synthetic *X-Delta-1* RNA persists throughout the neural plate stage, during the critical period for primary neurogenesis (data not shown). Embryos were fixed for 1 h at room temperature in MEMFA⁴⁵, stained overnight with X-gal²⁴, washed in MEMFA and stored in ethanol. Embryos were then processed for whole-mount *in situ* hybridization as described above. For immunohistochemistry, embryos

were stained first for X-gal staining, and then labelled with Islet-1 antibodies⁵. Antibody binding was revealed with a horseradish peroxidase-coupled secondary antibody and diaminobenzidine staining.

FIG. 4 Interfering with endogenous *X-Delta-1* activity causes a neurogenic phenotype. a, Extra neuronal cells in an embryo injected with *X-Delta-1*^{STU} (1.0 ng) and *lacZ* transcripts, and processed as above. Note high density and slightly broader domain of neuronal cells in the lateral stripe (arrow) on the injected side of the embryos. b, Similar embryo except that cell division was blocked from stage 10.5. Again, *X-Delta-1*^{STU} causes excessive neuron production (arrow).

METHODS. *X-Delta-1*^{STU} is a truncated construct lacking the last 139 amino acids of the intracellular domain of *X-Delta-1*, and was generated by cutting the CS2-*X-Delta-1* expression plasmid with *Stu*I and recircularizing. The resulting plasmid can be transcribed by SP6 to give an RNA encoding amino acids 1–583 of *X-Delta-1* plus an extra 12 amino acids derived from the vector polylinker. To block cell division, embryos were treated with

hydroxyurea and amphidicolin (HUA) using conditions described previously²⁴.



tion of RNA, restricted to a subset of the progeny of the injected blastomere. Each of the three stripes of *N-tubulin* expression can be reduced or eliminated independently when injected *X-Delta-1* transcripts are localized to the appropriate region of the neural plate. Thus, *N-tubulin* expression is inhibited in the portion of the neural plate where X-gal staining is most evident, that is, at the site of ectopic *X-Delta-1* transcripts, whereas nearby stripes of *N-tubulin* expression are not affected (Fig. 3a–c). With multiple injections, it is possible to achieve a broader distribution of RNA and to suppress primary neurogenesis in all three domains—medial, intermediate and lateral (Fig. 3e).

We confirmed that the loss of *N-tubulin* expression reflects the elimination of primary neurons by immunostaining for Islet-1 in injected embryos that had been left to develop to neural-tube stages. Antibody against Islet-1, a LIM-domain transcription factor, stains primary neurons in zebrafish³⁵ and *Xenopus* (data not shown). Upon sectioning, the number of cells stained for Islet-1 is conspicuously reduced on the side injected with *X-Delta-1* RNA (Fig. 3f). Averaged cell counts obtained from 8 embryos (15–56 sections per embryo) revealed a threefold reduction in the number of Islet-1-positive cells on the injected versus uninjected sides (0.39 versus 1.28 cells per section, respectively, $P < 0.05$). The neural tube is also thinner on this side (Fig. 3f), as expected from the absence of primary neurons, but its overall morphology is approximately normal, indicating that the effects of ectopic *X-Delta-1* on neurogenesis are specific.

By activated X-Notch-1. If *X-Delta-1* suppressed neurogenesis by activating X-Notch-1, the effects of *X-Delta-1* overexpression should be mimicked by a constitutively activated form of X-Notch-1. To test this, we injected RNA coding for either of two truncated versions of *X-Notch-1* (*NotchΔE* and *Notch ICD*²⁴) into one blastomere at the two-cell stage. Each construct lacks an extracellular domain and has been shown to be constitutively active^{10,24,26,36–38}. In *Xenopus*, these mutant Notch proteins cause severe developmental defects, including an increase in the amount of neuroepithelial tissue, as indicated by staining for the general neuroepithelial marker N-CAM^{24,25}. However, we find that injection of *NotchΔE* or *Notch ICD* transcripts inhibits primary neurogenesis: on the injected side *N-tubulin*-expressing cells at the neural plate stage are absent or consistently reduced in number (Fig. 3g, h; 55 embryos examined).

The similar effects on neurogenesis of *X-Delta-1* overexpression and constitutive Notch activation support the view that X-Delta-1 indeed functions as a ligand for X-Notch-1. The more widespread consequences of the constitutive X-Notch-1 signalling may be due to effects on tissues where neither *X-Delta-1* nor *X-Notch-1* is normally expressed. Also, Notch is thought to be a multifunctional receptor^{39,40}, so the truncated X-Notch-1 may activate other signalling pathways in addition to those normally dependent on X-Delta-1.

Endogenous *X-Delta-1* RNA expression is also reduced in *NotchΔE*-injected embryos, indicating that Notch activation negatively regulates *X-Delta-1* (Fig. 3i). This result was seen in

17/17 cases, whereas injecting *lacZ* transcripts causes no change of *X-Delta-1* expression (Fig. 3j; 9/9 cases). As we discuss below, this suggests that a competitive feedback mechanism may operate in embryos to accentuate and preserve the contrast between a neuronal precursor, expressing *X-Delta-1*, and its non-neuronal neighbours.

An antimorphic X-Delta-1 construct

If *X-Delta-1* overexpression causes a decrease in the number of primary neurons, a reduction of *X-Delta-1* activity would be predicted to cause an overproduction. In the course of analysing the activities of truncated X-Delta-1 proteins, we discovered that a construct lacking the intracellular domain, *X-Delta-1^{STU}*, behaves as an antimorph, giving a phenotype opposite to that produced by *X-Delta-1* overexpression, presumably by interfering with endogenous *X-Delta-1* activity. Unilateral injection of *X-Delta-1^{STU}* transcripts leads to a dramatic increase in the number of neuronal cells on the injected side of the embryo, as judged by the number of *N-tubulin*-staining cells (Fig. 4a and Table 1). Whereas neurons normally arise as scattered cells amid a non-neuronal population (for example, Fig. 2b), entire fields of cells express *N-tubulin* in embryos injected with *X-Delta-1^{STU}* (Fig. 4a). Co-injecting wild-type *X-Delta-1* with *X-Delta-1^{STU}* transcripts prevents the neuronal overproduction, and leads instead to suppression of neurogenesis as found for X-Delta-1 alone (Table 1). This confirms that *X-Delta-1^{STU}* acts by interfering with endogenous *X-Delta-1* signalling. *X-Delta-1^{STU}* might interfere with signal presentation by binding to endogenous X-Delta-1 to create an inactive complex; alternatively, it might compete with endogenous signal for the receptor on neighbouring cells, thereby preventing them from receiving the signal.

To distinguish effects on cell commitment from effects on cell proliferation, we incubated *X-Delta-1^{STU}*-injected embryos in hydroxyurea and amphidicolin to block cell division²⁴. There is again striking overproduction of neurons (Fig. 4b), showing that it is the commitment to a neuronal fate that is regulated by *X-Delta-1*.

Discussion

We have described *X-Delta-1*, a *Xenopus* homologue of *Drosophila Delta*. The gene is expressed during primary neurogenesis when commitment to a neuronal fate is thought to occur, and we have examined its function in this process. Our findings argue strongly that *X-Delta-1* regulates primary neurogenesis in the *Xenopus* embryo through a mechanism of lateral inhibition so as to limit the proportion of cells within the neural plate that actually become primary neurons.

We have shown that *X-Delta-1* is expressed in scattered cells in the domains of the neural plate where primary neuronal precursors are being generated (Figs 1 and 2). The expression is transient and consistently anticipates expression of *N-tubulin*, an early marker for primary neurons^{33,34}. Precise correspondence between the expression pattern of *X-Delta-1* initially and that of

TABLE 1 Effects of *X-Delta-1* and *X-Delta-1^{STU}* on primary neurons

No. of embryos with:	Embryos injected with:								<i>lacZ</i> alone 0.2 ng
	<i>X-Delta-1</i>				<i>X-Delta-1^{STU}</i>			<i>X-Delta-1</i> + <i>X-Delta-1^{STU}</i>	
	1.0 ng	0.3 ng	0.16 ng	0.08 ng	1.0 ng	0.3 ng	1.0 ng; HU	1.0 ng + 0.3 ng	
Fewer <i>N-tubulin</i> -expressing cells	65	17	16	30	0	0	0	10	5
<i>N-tubulin</i> expression unaffected	5	0	1	7	2	0	0	1	22
More <i>N-tubulin</i> -expressing cells	0	0	0	0	27	5	11	1	5
Total embryos analysed	70	17	17	37	29	5	11	12	34

Embryos were injected at the two-cell stage with *lacZ* RNA as a tracer and different concentrations of *X-Delta-1* and/or *X-Delta-1^{STU}* RNA. At the neural-plate stage, embryos were processed for X-gal staining and *N-tubulin* expression using whole-mount *in situ* hybridization. Embryos with X-gal staining in the neural plate were scored by comparing numbers of *N-tubulin*-expressing cells on the control and injected sides. Some embryos were also treated with hydroxyurea and amphidicolin (HU)²⁴.

N-tubulin slightly later suggests that the cells expressing *X-Delta-1* are the prospective primary neurons. This point is corroborated by our studies in the chick showing that cells expressing *C-Delta-1* in the central nervous system (CNS) are indeed post-mitotic neuronal precursors²⁷.

The function of *X-Delta-1* expression by these cells is demonstrated by two complementary findings. Ectopic *X-Delta-1* expression suppresses primary neurogenesis, showing that *X-Delta-1* has an inhibitory action, and that cells that would normally become primary neurons are susceptible to inhibition. Conversely, when we interfere with the action of endogenous *X-Delta-1* by expression of *X-Delta-1^{STU}*, the population of primary neurons increases dramatically, as though an inhibition has been removed (Fig. 4). As the cells normally expressing *X-Delta-1* appear to be the future primary neurons themselves, we presume that these must be the source of the inhibitory signal that prevents neighbouring cells—all of which express *X-Notch-1*—from taking the neuronal pathway (Fig. 2c). Thus, *X-Delta-1*'s role in primary neurogenesis is to mediate lateral inhibition so that only a limited proportion of the cells with potential to become primary neurons will actually do so.

The increase in cells undergoing neuronal differentiation in embryos injected with *X-Delta-1^{STU}* is confined to the domains of primary neurogenesis; the boundaries of these domains appear largely unchanged. Thus, the role of *X-Delta-1* appears to be confined to a domain in the neural plate that resembles a proneural domain of the type seen in *Drosophila*, within which most or all cells are competent to generate primary neurons but the majority are normally inhibited from doing so. Although we have not followed the fate of the inhibited, uncommitted cells, they presumably participate in subsequent waves of neurogenesis. Indeed, *X-Delta-1* expression is switched off in the prospective neurons as they begin to differentiate, and this should release neighbouring cells from inhibition so that further neurons can be produced, as occurs in *Drosophila*^{3,41}. *X-Delta-1* (and its chick counterpart) is also transiently expressed in subsequent sets of neuronal precursors during later neurogenesis (unpublished results, and ref. 27). Thus, lateral inhibition can serve to maintain a population of proliferating uncommitted cells so as

to allow continuing production of neurons and glial cells in the developing vertebrate CNS.

X-Delta-1 includes an N-terminal domain that is homologous to the putative Notch-binding domain of *Drosophila* Delta and Serrate^{13,27,29}, suggesting that *X-Delta-1* functions by binding to a Notch family member. Among the several *Notch* homologues present in vertebrates, *X-Notch-1* is a strong candidate for encoding this receptor because it is expressed in the same proneural domains as *X-Delta-1*, although with a more uniform distribution. Moreover, constitutively active forms of *X-Notch-1* block the formation of primary neurons, mimicking the effects of *X-Delta-1* overexpression, as expected if *X-Delta-1* delivers inhibition by activating *X-Notch-1*. The same experiments show that activated Notch inhibits expression of *X-Delta-1* (Fig. 3i, j), creating a potential feedback loop that would reinforce contrasts between adjacent cells. Thus, *X-Delta-1* expression in a given cell would activate *X-Notch-1* in its neighbours, thereby inhibiting their *X-Delta-1* expression and decreasing their ability to inhibit the original signalling cell. Such feedback regulation is similar to the mechanism of Delta/Notch-dependent cell competition proposed to act in *Drosophila*^{9,42,43} and also in *C. elegans*, where activation of the *Notch* homologue *lin-12* represses expression of the *Delta* homologue *lag-2* (ref. 44).

Although we have focused on lateral inhibition during primary neurogenesis, *X-Delta-1* is also expressed at other sites and times of neurogenesis, including the anterior neural plate and neurogenic placodes (Fig. 1) and the later stages of neural tube development when secondary neurons are generated (unpublished data). Thus, lateral inhibition mediated by Delta/Notch signalling appears to be a general mechanism for regulating commitment to a neuronal fate during vertebrate neurogenesis (see also accompanying paper²⁷). The same mechanism may also operate to generate local diversity of cell fates in non-neural vertebrate tissues, where *Delta* and *Notch* genes are also expressed (unpublished results). Taken together with the evidence that Notch and Delta family members guide a great variety of cell fate decisions in *Drosophila* and *C. elegans*^{10,14}, our results suggest that the Delta/Notch signalling pathway is a universal device for controlling fine-grained patterns of cell differentiation in animal tissues. □

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1. Campos-Ortega, J. A. & Hartenstein, V. *The Embryonic Development of Drosophila melanogaster* (Springer-Verlag, Berlin and New York, 1985).
2. Doe, C. Q. *Development* **118**, 855–863 (1992).
3. Skeath, J. B. & Carroll, S. B. *FASEB J.* **8**, 714–721 (1994).
4. Artavanis-Tsakonas, S. & Simpson, P. *Trends Genet.* **7**, 403–408 (1991).
5. Doe, C. Q. & Goodman, C. S. *Dev. Biol.* **111**, 206–219 (1985).
6. Lehmann, R., Dietrich, U., Jimenez, F. & Campos-Ortega, J. A. *Wilhelm Roux Arch. dev. Biol.* **190**, 226–229 (1981).
7. Lehmann, R., Jimenez, F., Dietrich, W. & Campos-Ortega, J. A. *Wilhelm Roux Arch. dev. Biol.* **192**, 62–74 (1983).
8. Heitzler, P. & Simpson, P. *Cell* **64**, 1083–1092 (1991).
9. Fortini, M. E. & Artavanis-Tsakonas, S. *Cell* **75**, 1245–1247 (1993).
10. Muskavitch, M. *Dev. Biol.* **166**, 415–430 (1994).
11. Sternberg, P. W. *Curr. Biol.* **3**, 763–765 (1993).
12. Henderson, S. T., Gao, D., Lambie, E. J. & Kimble, J. *Development* **120**, 2913–2924 (1994).
13. Greenwald, I. *Curr. Opin. genet. Dev.* **4**, 556–562 (1994).
14. Kopan, R. & Weintraub, H. *J. Cell Biol.* **121**, 631–641 (1993).
15. Lardelli, M., Dahlstrand, J. & Lendahl, U. *Mech. Dev.* **46**, 123–136 (1994).
16. Lardelli, M. & Lendahl, U. *Exp. Cell Res.* **204**, 364–372 (1993).
17. Weinmaster, G., Roberts, V. J. & Lemke, G. *Development* **113**, 199–205 (1991).
18. Weinmaster, G., Roberts, V. J. & Lemke, G. *Development* **116**, 931–941 (1992).
19. Coffman, C., Harris, W. & Kintner, C. *Science* **249**, 1438–1441 (1990).
20. Bierkamp, C. & Campos-Ortega, J. A. *Mech. Dev.* **43**, 87–100 (1993).
21. Swiatek, P. J., Lindsell, C. E., Franco del Amo, F., Weinmaster, G. & Gridley, T. *Genes Dev.* **8**, 707–719 (1994).
22. Conlon, R. A., Requema, A. G. & Rossant, J. *Development* **121**, 1533–1545 (1995).
23. Coffman, C. R., Skoglund, P., Harris, W. A. & Kintner, C. R. *Cell* **73**, 659–671 (1993).
24. Kopan, R., Nye, J. S. & Weintraub, H. *Development* **120**, 2385–2396 (1994).

25. Nye, J. S., Kopan, R. & Axel, R. *Development* **120**, 2421–2430 (1994).
26. Henrique, D. et al. *Nature* **375**, 787–790 (1995).
27. Thomas, U., Speicher, S. A. & Knust, E. *Development* **111**, 749–761 (1991).
28. Tax, F. E., Yeagers, J. J. & Thomas, J. H. *Nature* **368**, 150–154 (1994).
29. Hartenstein, V. *Neuron* **3**, 399–411 (1989).
30. Hartenstein, V. *J. comp. Neurol.* **328**, 213–231 (1993).
31. Roberts, A. & Clarke, J. D. W. *Phil. Trans. R. Soc. B* **296**, 195–212 (1983).
32. Oswald, R., Richter, K. & Grunz, H. *Int. J. dev. Biol.* **35**, 399–405 (1991).
33. Richter, K., Grunz, H. & Dawid, I. B. *Proc. natn. Acad. Sci. U.S.A.* **85**, 8086–8090 (1988).
34. Korzh, V., Edlund, T. & Thor, S. *Development* **118**, 417–425 (1993).
35. Struhl, G., Fitzgerald, K. & Greenwald, I. *Cell* **74**, 331–345 (1993).
36. Lieber, T., Kidd, S., Alcamo, E., Corbin, V. & Young, M. W. *Genes Dev.* **7**, 1949–1965 (1993).
37. Roehl, H. & Kimble, J. *Nature* **264**, 632–635 (1993).
38. Rebay, I. et al. *Cell* **67**, 687–699 (1991).
39. Couso, J. P. & Martinez Arias, A. *Cell* **79**, 259–272 (1994).
40. Hartenstein, V. & Campos-Ortega, J. A. *Wilhelm Roux Arch. dev. Biol.* **193**, 308–325 (1984).
41. Simpson, P. et al. *Cold Spring Harb. Symp. quant. Biol.* **57**, 391–400 (1992).
42. Goriely, A., Dumont, N., Dambly-Chaudiere, C. & Ghysen, A. *Development* **113**, 1395–1404 (1991).
43. Wilkinson, H. A., Fitzgerald, K. & Greenwald, I. *Cell* **799**, 1187–1198 (1994).
44. Harland, R. M. *Meth. Cell Biol.* **36**, 685–695 (1991).
45. Turner, D. L. & Weintraub, H. *Genes Dev.* **8**, 1434–1447 (1994).

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The signature of conductance quantization in metallic point contacts

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AN electrical current passing through a perfectly smooth narrow constriction is carried by a finite number of quantized modes (analogous to those in a waveguide), each of which contributes $2e^2/h$ to the conductance¹. Conductance quantization has been observed in semiconductor devices containing a two-dimensional electron gas^{2,3}, where the width of the constriction is adjusted continuously by applying an electric field. A similar effect is expected to occur in three-dimensional metallic point contacts^{4,5}, and conductance steps of approximately $2e^2/h$ have recently been observed^{6–10}. But metallic point contacts do not change size continuously⁶, and thus the conductance steps reported in these earlier experiments might be attributable to discrete rearrangements of the atomic structure of the contact, rather than true conductance quantization¹¹. Here we use the fact that the degeneracy of the conduction modes of a three-dimensional point contact should result in a characteristic sequence of conductance values^{4,5} (some integer multiples of $2e^2/h$ are excluded) to distinguish the effects of conductance quantization from those of discrete variations in contact size in a break-junction experiment, confirming that conductance quantization does indeed occur.

We have used the technique of mechanically controllable break-junctions^{12,13} to create metallic contacts of adjustable size. This technique consists of breaking a fine metal wire of the material to be studied, in vacuum and at low temperature. The clean fracture surfaces are then brought back into contact, using a piezo-electric element for atomic-scale control of the distance. The wire ends are held fixed on the same substrate, which reduces the sensitivity to external vibrations of the inter-electrode distance to less than 10^{-3} Å.

In the present experiment, we measure the conductance in atom-sized contacts between metallic electrodes while pulling the electrodes apart; by increasing the voltage on the piezo element the size of the contact between the electrodes is reduced. Figure 1 shows a number of recordings of the conductance, G , of a contact as a function of the piezo voltage V_p . The metal studied here is sodium, which was selected because its electronic properties approximate those of a free electron gas. Just before losing contact between the electrodes, the conductance reproducibly equals the quantum unit $G_0 = 2e^2/h$. Other plateaus in the conductance, between abrupt steps, show a marked but not perfect coincidence with quantized values. Previous studies on the abrupt steps in metals¹³ and in semimetals¹⁴ have shown that these jumps result from atomic rearrangements in the contact area.

The following observations confirm that the same mechanism is responsible for the steps observed here. Sweeping V_p slowly forwards and backwards over a single step, we find that the steps either show hysteresis, or two-level fluctuations (TLFs). The TLFs are seen when V_p is held fixed at a step. The contact conductance then switches spontaneously between the values before and after the step. In fact, we observed that the smooth transition between G values of $1 G_0$ and $3 G_0$ (as seen in some of the traces) is the result of averaging such a TLF over a time (0.1–0.3 s) that is much longer than the fluctuation period (here ~ 1 ms). Changing V_p gradually inverts the dominant occupa-

tion of the states. As discussed previously¹³, both the TLFs and the hysteresis are the results of a switching between local potential minima for the atomic geometry of the contact. When the energy barrier between the states is high, hysteresis results. For lower barriers, thermal excitation induces TLF behaviour. It is possible to excite the TLFs to a higher fluctuation frequency by increasing the current through the contact. On the plateaus (Fig. 1) such fluctuations are absent; they are only observed at the steps. The fluctuation of the contact area is observed as TLFs in the conductance.

The differences between the experimental curves in Fig. 1 are attributed to variations in the atomic-scale geometry of the contacts. The contact size changes stepwise between the various atomic geometries, so the conductance curve as a function of contact size is sampled in discrete atom-sized steps. This complicates the study of conductance quantization. However, the common features of a large number of curves, such as those shown in Fig. 1, can be extracted by projecting all the curves onto the conductance axis, producing the histogram shown in Fig. 2a. This histogram of conductance values is constructed by dividing the conductance axis into equally spaced intervals of $\frac{1}{15} G_0$. In each interval, the number of points of the digitized $G(V_p)$ curves was counted. The result was summed for 105 individual recordings, measured on two samples. The histogram in Fig. 2a has a characteristic structure: there is a sharp peak at $1 G_0$, a peak of the same height slightly below $3 G_0$, and smaller peaks near 5 and 6 G_0 . Peaks at 2, 4 and 7 G_0 are absent.

This characteristic sequence of peaks finds a natural explanation in terms of quantization of the conductance in the contact.

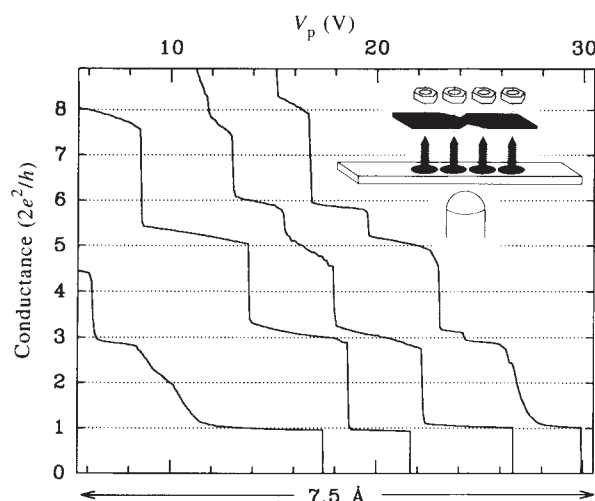


FIG. 1 Typical recordings of the measured conductance of atom-sized sodium contacts at 4.2 K. The electrodes are pulled apart by increasing the voltage across the piezo element, V_p (upper x-axis). The corresponding displacement is given on the lower axis. After each scan the electrodes were pressed together to form a large contact (resistance $< 100 \Omega$). The curves are displaced along the V_p -axis for clarity.

METHODS. The mechanically controllable break-junction technique¹² had to be modified to accommodate the highly reactive sodium metal. A schematic view of the sample mounting is shown in the inset. While immersed in paraffin oil, the sample (black horizontal bar) is pressed onto four 1-mm-diameter brass bolts, which are glued onto the isolated substrate (length ~ 20 mm). Current and voltage leads are fixed with nuts onto the bolts. A notch is cut into the sample at the centre. By bending the substrate at 4.2 K in vacuum, the sample is broken at the notch. Two fresh surfaces are exposed and the distance between them is controlled by changing the force on the substrate, employing a piezo element for fine control. The low-temperature environment ensures that the surfaces are not polluted by adsorbates. The purity of the material was at least 99.9%. The conductance was recorded using a standard low-frequency four-terminal lock-in technique, with a time constant of 0.1–0.3 s. Each scan was recorded in about 5 minutes.

We will first discuss the origin of these selected quantum numbers, and then propose an interpretation of the experiment which includes the role of the discrete atomic steps in the contact size.

For a point contact in a two-dimensional electron gas, the conductance may assume all integer numbers times G_0 . For a sufficiently symmetric, three-dimensional metallic contact, however, degeneracy of the modes excludes specific values from observation. (For example, if a particular mode is two-fold degenerate, it will contribute $2 G_0$ rather than G_0 to the conductance.) In Fig. 3, calculated curves^{4,5} for three values of the aperture angle α of a circularly symmetric contact are reproduced. In the limit $\alpha=0$, the contact is an infinitely long cylinder of radius a . The free-electron waves in the contact are described by $J_m(r) \exp(im\phi) \exp(ikz)$, where r , ϕ and z are the usual cylindrical coordinates and k is the wavevector in the direction of propagation. The z -coordinate is parallel to the symmetry axis of the contact. Quantization of the modes is determined by the condition that the Bessel function $J_m(r)$ is zero at the boundary. The zeros of the Bessel function, γ_{mn} , determine the energies, which are degenerate for the magnetic quantum numbers $+m$ and $-m$. For a continuously increasing contact diameter, the conductance increases stepwise each time new modes are found below the Fermi energy. Non-degenerate transitions occur when $m=0$; all other transitions are two-fold degenerate. As α increases to π , the sharply defined transitions gradually smear out. Further smearing of the steps results when roughness of the surface is introduced, and from disorder in the internal structure of the contact.

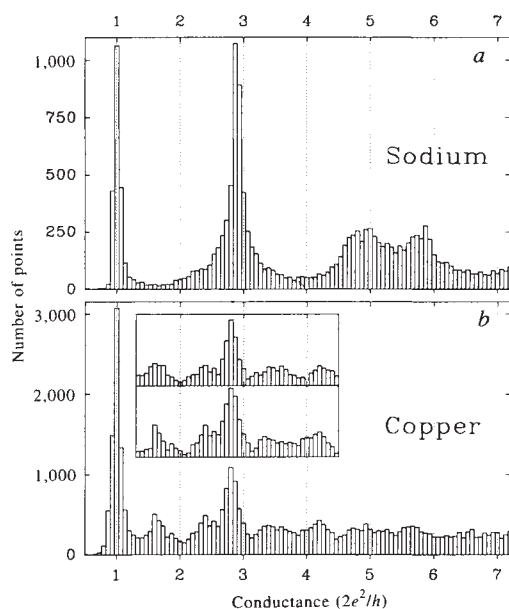


FIG. 2 *a*, Histogram of conductance values, constructed from measurements on sodium as shown in Fig. 1; each quantum conductance unit is divided into 15 equally spaced intervals. In each interval, the number of points of the digitized $G(V_0)$ curves was counted. The spectra were summed for 105 individual recordings, measured on two samples. The resulting series of peaks at $(1, 3, 5, 6, \dots)G_0$ forms the signature of conductance quantization. The histograms for each sample separately are very similar. The number of data points per ångström of electrode displacement was approximately the same for all scans. *b*, Histogram constructed from measurements on copper contacts, following the same procedure as for sodium. The spectrum is the sum of 90 scans on two different samples. Conductance quantization peaks at 1 and $3 G_0$ are seen, as well as additional peaks, probably resulting from stable atomic configurations of the contact. The insets show part of the spectra for the two samples separately, being the sum of 15 (top) and 75 (bottom) scans, illustrating the reproducibility of the smaller peaks.

The atomic rearrangements in our experiment give rise to finite steps in the contact size, of the order of the cross-section of an atom. For monovalent metals the density of atoms equals the density of conduction electrons, from which one may derive a general relation between the cross-sectional area of an atom, and the free-electron Fermi wavelength (λ_F) . One obtains $\pi r_a^2 = 0.907(\lambda_F/2)^2$ and $\pi r_a^2 = 0.960(\lambda_F/2)^2$ for body-centred cubic (b.c.c.) and face-centred cubic (f.c.c.) metals, respectively, with r_a being half the interatomic distance. The step size of the contact area in the experiment is, therefore, expected to be of the order of $(\lambda_F/2)^2$. For small opening angles α (Fig. 3), the conductance values probed by the contact will coincide with high probability with the quantized sequence. In the sodium experiment, α appears to be small enough for this to occur. The mechanics of fracture of atomic-scale contacts at low temperatures¹⁵ is such that a neck forms, which elongates and finally breaks when the contact is reduced to a single atom. The neck formation ensures small values of the aperture angle α and approximate cylindrical symmetry. For the quantization to be observed it is furthermore necessary that the atomic-scale corrugation of the surface is smoothed out. This smoothing is expected for free-electron metals, such as sodium, where the electron energy-level structure of clusters as small as a few atoms is experimentally found to agree with predictions for a spherical box¹⁶.

The peaks in Fig. 2*a* appear at values slightly lower than the theoretical quantized values; this shift increases as the contact radius increases. Such shifts may arise from a series resistance of $\sim 100 \Omega$ due to the metal leads near the contact. On the basis of this series resistance we estimate a mean free path l_e of 100 Å, which is reasonable in view of the mechanical deformations resulting from the contact formation process.

The conductance quantization is smeared out when, for example, α is close to π , or when the mean free path becomes comparable to the contact diameter. Figure 2*b* shows a histogram of conductance values measured for copper. Comparing this result to the sodium experiment, we observe that the peak at $1 G_0$ occurs again with copper, the peak at $2 G_0$ is again absent, but the peak at $3 G_0$ is less pronounced for copper, and peaks near 5 and $6 G_0$ are not resolved. The difference between the results for copper and sodium may arise from a difference in electronic properties. The free-electron character is less pronounced for copper than it is for sodium, as is also observed in the cluster experiments¹⁶. Also, differences in mechanical proper-

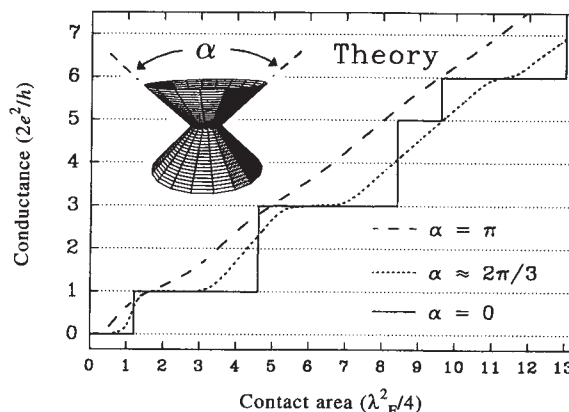


FIG. 3 Calculated conductance for a free electron gas and a cylindrically symmetrical contact, as a function of the contact area for three different aperture angles: $\alpha=0$, $2\pi/3$ and π . The horizontal axis corresponds to the contact area in units $(\lambda_F/2)^2$ (see text), which is assumed to be a continuous parameter in the model calculation. The geometry of the contact is sketched in the inset, in which the aperture angle α is defined. (After Bogachev *et al.*⁴ and Torres *et al.*⁵.)

ties might result in shorter necks, which can be represented by a larger α . These effects would result in smearing of the transitions between the quantized levels in Fig. 3. Probing a strongly smeared conductance curve at discrete values of the contact area will show little sign of the quantization, but may produce peaks in the histogram corresponding to stable atomic geometries. Indeed, there are reproducible peaks near 1.6, 2.4, 3.4 and 4.2 G_0 (Fig. 2b) and we speculate that these are due to preferred atomic configurations of the copper contact.

Our experiments show that the variation of the conductance in atomic-scale metal contacts of varying size is therefore determined both by quantization of the conductance and by the discreteness of the contact size. The characteristic sequence of quantum numbers observed in the conductance histogram for sodium gives compelling evidence for the dominant presence of quantized conductance channels, which is explained by its free-electron properties, and is only observable after averaging over a large number of atomic geometries. Even for a d -metal such as copper, the quantization pattern can be recognized in the lower range of the conductance, but the role of atomic geometries is more prominent. Having observed quantization of the conductance, it is of interest to study the effect of these quantum-size phenomena on other physical properties, such as magnetic

impurity scattering and superconductivity. Such experiments are already being performed^{17,18}. □

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1. Beenakker, C. W. J. & van Houten, H. in *Solid State Physics Vol. 44* (eds Ehrenreich, H. & Turnbull, D.) (Academic, New York, 1991).
2. van Wees, B. J. et al. *Phys. Rev. Lett.* **60**, 848–850 (1988).
3. Wharam, D. A. et al. *J. Phys. C* **21**, L209–L214 (1988).
4. Bogachev, E. N., Zagorskii, A. N. & Kulik, I. O. *Soviet J. Low Temp. Phys.* **16**, 796–800 (1990).
5. Torres, J. A., Pascual, J. I. & Sáenz, J. J. *Phys. Rev. B* **49**, 16581–16584 (1994).
6. Krans, J. M. et al. *Phys. Rev. B* **48**, 14721–14724 (1993).
7. Agrait, N., Rodrigo, J. G. & Vieira, S. *Phys. Rev. B* **47**, 12345–12348 (1993).
8. Olesen, L. et al. *Phys. Rev. Lett.* **72**, 2251–2254 (1994).
9. Pascual, J. I. et al. *Phys. Rev. Lett.* **71**, 1852–1855 (1993).
10. Olesen, L. et al. *Phys. Rev. Lett.* **74**, 2147 (1995).
11. Krans, J. M. et al. *Phys. Rev. Lett.* **74**, 2146 (1995).
12. Muller, C. J., van Ruitenbeek, J. M. & de Jongh, L. J. *Physica C* **191**, 485–504 (1992).
13. Muller, C. J., van Ruitenbeek, J. M. & de Jongh, L. J. *Phys. Rev. Lett.* **69**, 140–143 (1992).
14. Krans, J. M. & van Ruitenbeek, J. M. *Phys. Rev. B* **50**, 17659–17661 (1994).
15. Agrait, N., Rodrigo, J. G., Sirvent, C. & Vieira, S. *Phys. Rev. B* **48**, 8499–8501 (1993).
16. de Heer, W. A. *Rev. Mod. Phys.* **65**, 611–676 (1993).
17. Yanson, I. K. et al. *Phys. Rev. Lett.* **74**, 302–305 (1995).
18. van der Post, N., Peters, E. T., Yanson, I. K. & van Ruitenbeek, J. M. *Phys. Rev. Lett.* **73**, 2611–2613 (1994).

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Synthesis and characterization of carbide nanorods

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THE properties and potential applications of carbon nanotubes filled with other materials have aroused much speculation^{1–5}. Strategies for filling nanotubes include *in situ* growth in an arc reactor using metal/carbon composites^{2,5} and the capillarity-driven filling of open nanotubes using liquid reagents^{3,4}. Here we report an alternative approach to the synthesis of nanoscale structures based on nanotubes, in which the tubes are converted to carbide rods by reaction with volatile oxide and/or halide species. In this way we have been able to prepare solid carbide nanoscale rods of TiC, NbC, Fe₃C, SiC and BC_x in high yield with typical diameters of between 2 and 30 nm and lengths of up to 20 μ m. Preliminary studies show that these rods share the properties of the bulk materials (such as magnetism and superconductivity), suggesting that they might allow the investigation of the effects of confinement and reduced dimensionality on such solid-state properties. These carbide nanorods might also find technological applications in nanostructured composite materials.

Our preparation of carbide nanorods involves the reaction of carbon nanotubes with volatile metal or non-metal complexes (Fig. 1). The carbon nanotubes used in these vapour–solid reactions were obtained from metal-catalysed growth using ethylene and hydrogen⁶. This procedure yields relatively pure nanotube samples compared with arc-discharge methods^{7–9}, although the nanotubes exhibit poor crystallinity (Fig. 2a). Previous studies have also shown that SiO vapour can be used to convert carbon fibres¹⁰ and nanotubes¹¹ to SiC rods, although the sizes of these SiC products were typically much larger than the carbon precursor¹¹. In our studies discussed below, the diameters of the solid nanorods are similar to the starting diameters of the

nanotube reactants and significantly smaller than reported previously^{10,11}. Furthermore, our general approach (Fig. 1) has been exploited to prepare a wide range of chemically distinct carbide materials.

The morphology and structure of the products obtained from the reaction of TiO and carbon nanotubes at 1,375 °C are shown in Fig. 2. Transmission electron microscopy (TEM) images of the reaction product (Fig. 2b–d) show both straight and smoothly curved, solid rod-like structures that are distinct from the irregularly curved and hollow carbon nanotube reactant (Fig. 2a). These images also show that the diameters of the rod-like products are similar to that of the carbon nanotubes, 2–30 nm, and that the lengths typically exceed 1 μ m. Energy dispersive X-ray fluorescence and electron energy-loss spectroscopy measurements demonstrate that these nanorods contain only titanium and sp^3 -hybridized carbon, and thus are consistent with the conversion of the carbon nanotubes into titanium carbide (TiC).

This formulation is further established by structural analyses. Powder X-ray diffraction (XRD) measurements on nanorod samples produced using either TiO or Ti + I₂ show diffraction peaks that can be indexed to the known cubic, rock-salt structure of TiC with no evidence of either graphitic (nanotube), Ti-metal or Ti-oxide peaks present. The measured lattice constant, $a = 4.326$ Å, is consistent with a stoichiometry TiC_x, with $x \approx 1$ (ref. 12). TEM and electron diffraction studies of single nano-

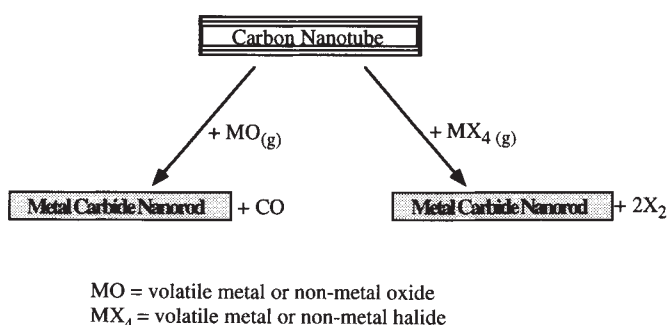


FIG. 1 Reaction scheme used to prepare metal carbide nanorods.

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rods (Fig. 2c–f) provide further insight into the structures of these TiC materials. In general, we observe smooth (Fig. 2c), regular saw-tooth (Fig. 2d) and irregularly faceted (Fig. 2e) nanorod morphologies; the TiC nanorods also appear to be single crystals with a very low density of stacking faults. Convergent beam electron diffraction patterns recorded along the $\langle 111 \rangle$ zone axis perpendicular to the axis of the smooth nanorod (Fig. 2c) exhibit a lattice constant and six-fold symmetry corresponding to the (111) planes of cubic TiC. These data imply that the axis of the smooth TiC nanorods is $[110]$. Similar studies of the saw-tooth nanorod (Fig. 2d) demonstrate that the rod axis is also $[110]$ for this morphology. The $[110]$ direction is not unique for the TiC nanorods. In the irregularly faceted nanorods (Fig. 2e, f), high-resolution TEM and electron diffraction demonstrate that the rod axis is $[111]$, and further show that the TiC nanorods contain single-crystal domains often exceeding $1 \mu\text{m}$ in length. We note that the saw-tooth morphology has obvious advantages for some applications (for example, composites), although more work is clearly needed to understand and control its growth.

Our approach to carbide nanorod synthesis seems to be general. For example, structural and composition analyses of the material produced from the reaction of carbon nanotubes with

Si and I_2 are consistent with the formation of silicon carbide (SiC) nanorods. TEM images show that the SiC nanorods produced from this reaction are relatively straight, solid rods (Fig. 3a, b). The diameters of the SiC rods produced in the Si + I_2 reaction at $1,200^\circ\text{C}$ are typically 2–20 nm (similar to the diameters of the carbon nanotube reactants), and have lengths of the order of $1 \mu\text{m}$. These diameters are significantly smaller than reported¹¹ recently for the reaction of SiO with impure nanotubes at $1,700^\circ\text{C}$. X-ray diffraction patterns from bulk samples were indexed to the zinc blende β -SiC structure and show no evidence of other crystalline impurities. Transmission electron microscopy shows that these SiC nanorods possess a high density of planar defects (Fig. 3b–d) in contrast to the near-single-crystal TiC nanorods. We suggest that the defects in $[111]$ oriented rods (Fig. 3b, d) are rotational twin stacking faults, by analogy with previous observations made on larger SiC whiskers^{13,14}. The rod axes lie along the $[111]$ direction in all of the nanorods prepared at $1,300$ – $1,400^\circ\text{C}$ using SiO as the volatile silicon reactant, but at the lower Si + I_2 reaction temperatures ($1,100$ – $1,200^\circ\text{C}$) this direction is not unique. A high-resolution TEM image of one 7-nm-diameter SiC nanorod (Fig. 3c) produced at $1,200^\circ\text{C}$ shows that the rod axis lies along the $[100]$ direction, although small defect regions with a $[111]$ direction are also present.

FIG. 2 TEM images of carbon nanotubes (a) and TiC nanorods (b–f). The nanotubes are imaged as irregular dark lines with light (hollow) centres, whereas the nanorods appear uniform (solid) across the rod axis. Low-resolution images (b) show that the nanorods grow with smooth and rough morphologies. The rod axes for these different morphologies were determined from electron diffraction and high-resolution images of single nanorods and are indicated with arrows in the images c–f. The regular sawtooth morphology (d) has a $[110]$ rod axis and a sawtooth angle of 109° . The irregular facets seen in the rods with a $[111]$ axis (e) were found in high-resolution lattice plane images (f) to lie along the $[111]$ direction. Variations in contrast seen along the nanorod axes (b–d) are due to orientation variations of the nanorods on the TEM supports. The insets in c–e correspond to electron diffraction patterns recorded along zone axes perpendicular to the rod axes (see text). The scale bars in a–e correspond to 20 nm. The carbon nanotubes were grown at 760°C using an ethylene/hydrogen feedstock and a supported iron catalyst⁶. The TiC nanorods were obtained from the reaction of carbon nanotubes and TiO at $1,375^\circ\text{C}$ for 12 h. The nanorods were easily dispersed in organic solvents, unlike carbon nanotubes⁶.

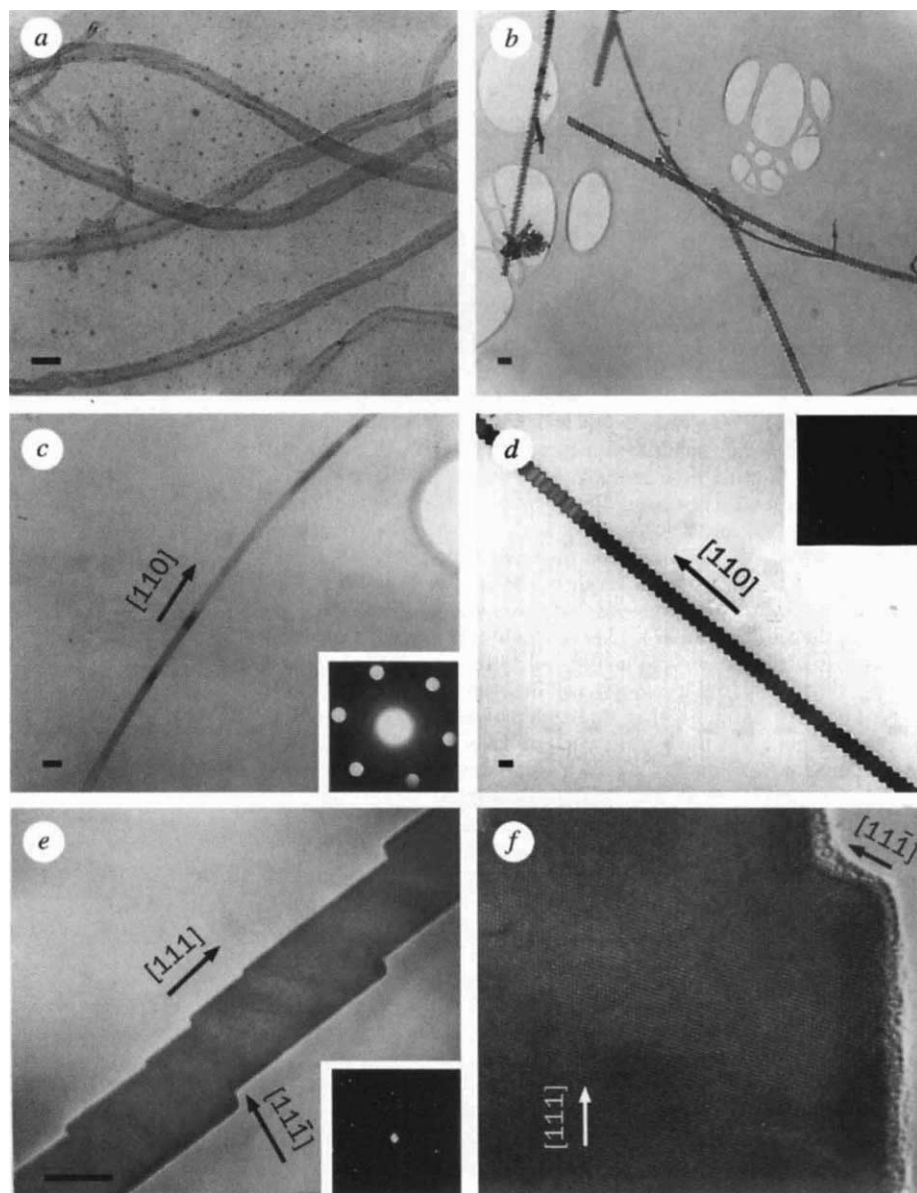


TABLE 1 Summary of the structure and properties of carbide nanorods

Carbide nanorod	Metal reactant* (reaction temp., °C)	Nanorod structure†	Properties‡
TiC	TiO (1,300–1,400) Ti + I ₂ (1,200–1,300)	Single crystal	Metal
NbC	Nb + I ₂ (≥ 700)	Polycrystalline	Superconductor
Fe ₃ C	FeCl ₃ (1,350)	Amorphous	Ferromagnetic
SiC	Si + I ₂ (1,100–1,200) SiO (1,300–1,450)	Single crystal	Semiconductor
BC _x	B ₂ O ₃ (1,400)	Polycrystalline	Insulator

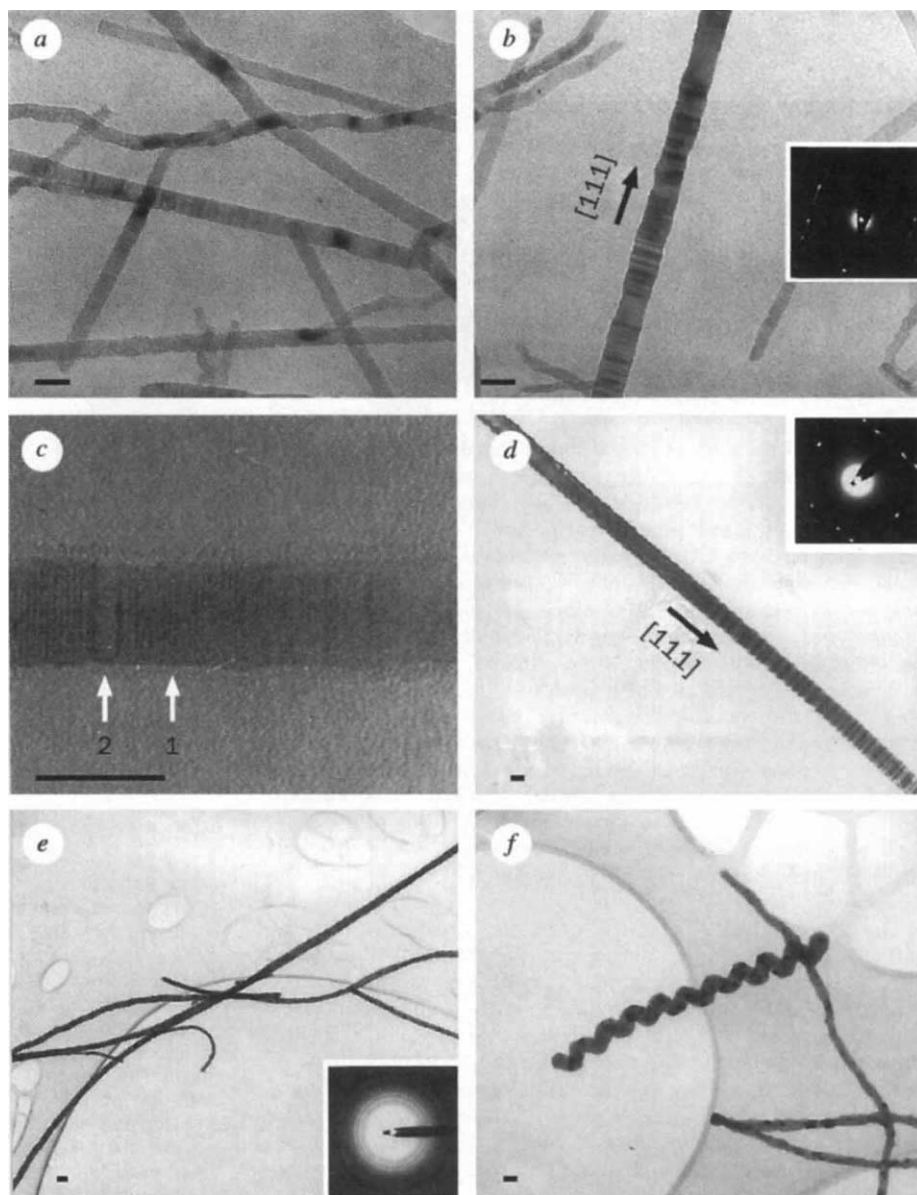
* All reactions (except for the case of boron carbide) were carried out in quartz tubes sealed under vacuum with the carbon nanotubes and metal reactants spatially separated. Boron carbide was formed in a flow reactor (Ar carrier) with B₂O₃ generated *in situ* by the reaction of boron with TiO₂¹⁹. Titanium was not detected in the resulting boron carbide nanorods. Phases were identified from structure and composition data. For the boron carbide nanorods, the data was most consistent with B₁₃C₂, although solid B₄C cannot be ruled out.

† The physical dimensions of the nanorods were similar: diameters of 2–30 nm and lengths typically >1 µm.

‡ The superconducting ($T_c \approx 9$ K; superconducting fraction ~70%) and ferromagnetic character of the NbC and Fe₃C nanorods were determined directly from d.c. magnetization measurements. The bulk T_c of NbC is ~12 K. The properties of the TiC, SiC and BC_x nanorods are inferred from the bulk materials.

Results from the synthesis of other metal carbides using our approach are summarized in Table 1. For example, the reaction of Nb metal and I₂ with carbon nanotubes yields NbC nanorods (Fig. 3e,f). X-ray diffraction data show that this reaction results in the complete conversion of the nanotubes into the cubic, rock-salt phase of NbC. TEM images and selected-area electron diffraction show that the NbC nanorods produced in these reactions are polycrystalline with morphologies (Fig. 3e,f) similar to the carbon nanotube starting materials. Significantly, these polycrystalline nanorods are also found to be superconducting, as is bulk NbC (see Table 1). The polycrystalline structure is probably due to the relatively low reaction temperature (730 °C), and thus it should be possible in the future to optimize the growth conditions and produce single-crystal NbC nanorods. The present reaction conditions can, however, yield unique morphologies, such as helical nanorods (Fig. 3f), that may also exhibit novel properties. These helical nanorods are believed to result from a direct conversion of helical carbon nanotubes produced in the catalytic growth process. Preliminary reactions between carbon nanotubes and FeCl₃ have been found to yield ferromagnetic Fe₃C nanorods (Table 1). Although these materials appear to be amorphous, we believe that the results are significant because the nanorods are magnetic.

FIG. 3 TEM images of SiC nanorods (a–d) and NbC nanorods (e–f). The SiC nanorods exhibit a high density of striations, which correspond to stacking faults, perpendicular to the rod axes. Selected area electron diffraction patterns recorded on samples with striations perpendicular to the [111] direction (b, d) exhibit extra diffraction spots along the $\langle 110 \rangle$ zone axis that are characteristic of twinning^{13,14}. A high-resolution lattice image (c) also shows a SiC nanorod with a primary rod axis direction of [100] (region labelled '1') and growth defects consisting of several unit cells oriented along [111] (region 2). The small dark and light regions in the NbC samples (e, f) correspond to individual crystal grains in these polycrystalline nanorods. The insets in b and d correspond to electron diffraction patterns recorded along zone axes perpendicular to the rod axes. Selected area diffraction of the NbC (inset e) shows a ring structure characteristic of a polycrystalline sample; the diffraction pattern was indexed to pure cubic NbC. The scale bars in a, b and d–f correspond to 20 nm; the bar in c corresponds to 10 nm. The SiC nanorods shown in a–c were obtained from the reaction of carbon nanotubes with Si and I₂ at 1,200 °C for 2 h. The SiC nanorod shown in d was obtained from the reaction of nanotubes and SiO in a vacuum sealed quartz tube at 1,400 °C for 1 h. The NbC nanorods were obtained from the reaction of nanotubes with Nb and I₂ at 730 °C for 35 h.



The growth of TiC and SiC whiskers that have a crystalline, rod-like structure have been studied previously because these materials represent attractive reinforcing additives for metal and ceramic matrix composites^{13–17}. The diameters of these whiskers have been typically >1,000 nm, although diameters as small as 100 nm have been observed. The carbide nanorods reported here have significantly smaller diameters, typically in the range 2–30 nm, and thus represent a major step in size reduction (comparable to the production of carbon nanotubes⁵ compared with carbon fibres¹⁸). We are not aware of carbide whiskers analogous to the other carbide nanorods prepared in our studies (Table 1). Our data can be explained in part by a mechanism involving template-mediated growth, whereby carbon nanotubes define the diameter of the product carbide nanorods formed following reaction with a volatile species^{10,11}. The similarity of the nanorod average diameters (and morphologies in the case of polycrystalline NbC) to that of the starting nanotubes is consistent with this proposal, but we cannot at present rule out contributions from catalytic growth and/or sintering of small tubes. Although a deeper understanding of the nanorod growth mechanism is clearly needed, we note that template-mediated growth could in principle produce carbide nanorods of any stable metal carbide.

We believe that these materials offer exciting opportunities for both fundamental research and technological applications. The wide range of nanorod properties and morphologies suggests that these materials may represent important building blocks for nanostructures. For instance, current flowing through our

helical, metallic nanorods would lead to a modulated magnetic field that might be exploited for sensing or manipulation. The small diameters and range of chemical compositions available for these carbide nanorods could also make them useful as chemically specific reinforcements in metal and ceramic matrix composites, as well as perhaps an ideal structure with which to pin vortices in high-temperature superconductors. □

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1. Ruoff, R. S. *Nature* **372**, 731–732 (1994).
2. Guerret-Piecourt, C., Le Bouar, Y., Loiseau, A. & Pascard, H. *Nature* **372**, 761–764 (1994).
3. Tsang, S. C., Chen, Y. K., Harris, P. J. F. & Green, M. L. H. *Nature* **372**, 159–162 (1994).
4. Ajayan, P. M. & Iijima, S. *Nature* **361**, 333–334 (1993).
5. Seraphin, S., Zhou, D., Jiao, J., Whithers, J. C. & Loutfy, R. *Nature* **362**, 503 (1993).
6. Snyder, C. E. et al. *Int. Patent* WO 89/07163 (1989).
7. Iijima, S. *Nature* **354**, 56–58 (1991).
8. Ebbesen, T. W. & Ajayan, P. M. *Nature* **358**, 220–222 (1992).
9. Colbert, D. T. et al. *Science* **266**, 1218–1222 (1994).
10. Okada, K. & Nakajima, K. *Eur. Patent Appl.* EP 60388 A2 (1993).
11. Zhou, D. & Seraphin, S. *Chem. Phys. Lett.* **222**, 233–238 (1994).
12. Storms, E. K. *The Refractory Carbides 1–17* (Academic, New York, 1967).
13. Wang, L., Wada, H. & Allard, L. F. J. *Mater. Res.* **7**, 148–163 (1992).
14. McMahon, G., Carpenter, G. J. C. & Malis, T. F. J. *Mater. Sci.* **26**, 5655–5663 (1991).
15. Verma, A. R. & Krishna, P. *Polymorphism and Polytypism in Crystals* (Wiley, New York, 1966).
16. Wokulski, Z. & Wokulska, K. J. *Cryst. Growth* **62**, 439–446 (1983).
17. Tamari, N. & Kato, A. J. *Cryst. Growth* **46**, 221–237 (1979).
18. Speck, J. S., Endo, M. & Dresselhaus, M. S. J. *Cryst. Growth* **94**, 834–848 (1989).
19. Lundstrom, T. in *Boron and Refractory Borides* (ed. Matkovich, V. I.) 351–376 (Springer, New York, 1977).

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An efficient prebiotic synthesis of cytosine and uracil

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IN contrast to the purines^{1–3}, the routes that have been proposed for the prebiotic synthesis of pyrimidines from simple precursors give only low yields. Cytosine can be synthesized from cyanoacetylene and cyanate^{4,5}; the former precursor is produced from a spark discharge in a CH₄/N₂ mixture^{4,5} and is an abundant interstellar molecule⁶. But this reaction requires relatively high concentrations of cyanate (>0.1 M), which are unlikely to occur in aqueous media as cyanate is hydrolysed rapidly to CO₂ and NH₃. An alternative route that has been explored⁷ is the reaction of cyanoacetaldehyde (formed by hydrolysis of cyanoacetylene⁸) with urea. But at low concentrations of urea, this reaction produces no detectable quantities of cytosine⁷. Here we show that in concentrated urea solution—such as might have been found in an evaporating lagoon or in pools on drying beaches on the early Earth—cyanoacetaldehyde reacts to form cytosine in yields of 30–50%, from which uracil can be formed by hydrolysis. These reactions provide a plausible route to the pyrimidine bases required in the RNA world⁹.

The previous lack of plausible prebiotic syntheses of cytosine and uracil has led some authors to suggest that other bases were used in the first genetic material, such as the pyrimidines isocytosine and diaminopyrimidine¹⁰, urazole and guanazole¹¹ and purines alone^{12,13}. But the presence of pyrimidines in the Murchison meteorite at one-fifth the concentration of purines^{14,15} suggests that an efficient prebiotic synthesis of pyrimidines should be possible. Ferris *et al.*⁷ showed that cyanoacetaldehyde reacts with guanidine to produce diaminopyrimidine, but only with yields of 0.1–2%. Diaminopyrimidine can serve as a source of uracil by hydrolysis, but this provides only very low

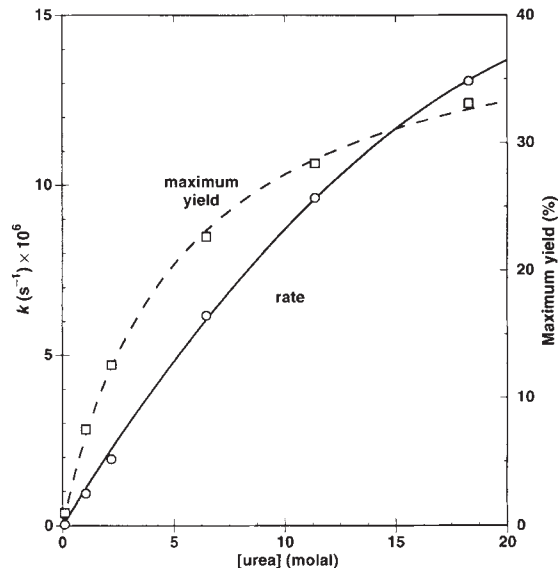


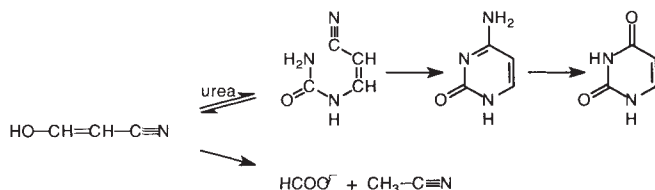
FIG 1 Rate constants and maximum yield for cytosine formation at 100 °C from cyanoacetaldehyde (10⁻³ M) and urea. These pseudo-first-order rate constants are based on the initial rates after 5 h. The maximum yield is the sum of cytosine and uracil which is formed by hydrolysis of cytosine (40% after 120 h). The cyanoacetaldehyde was prepared, from isoxazole (Aldrich) and the concentration determined spectrophotometrically using a molar absorptivity of 15,200 M⁻¹ cm⁻¹ at 248 nm for the anion⁷. The standard pyrimidines were obtained from Sigma. The HPLC analysis was performed with a Beckman model 110B HPLC system using a YMC ODS-AQ analytical reversed phase column and a Kratos absorbance detector set at 260 nm. The mobile phase was 0.1 M pH 4.8 sodium phosphate at 1 ml min⁻¹. The cytosine and uracil were identified by HPLC elution time, co-injection of a known sample, ultraviolet absorption spectra and proton NMR (GE 300 MHz).

yields of cytosine. Ferris *et al.*⁷ detected no cytosine formed from the reaction of cyanoacetaldehyde with urea. Other prebiotic syntheses of pyrimidines include ultraviolet-induced dehydrogenation of dihydrouracil on silica¹⁶ and the formation of orotic acid in low yield from cyanide polymerization¹⁷.

Because urea is a well established prebiotic compound^{18, 20} and is very soluble (the mole fraction²¹ is given by $\log X_2 = 1.468 - 609.8/T$), we investigated the very concentrated solutions that could be achieved in a drying lagoon or beach. Solutions of cyanoacetaldehyde and urea were heated in sealed ampoules in thermostated tube heaters and the pyrimidines analysed by high-performance liquid chromatography (HPLC). The rate constants for the reaction are shown for a number of urea concentrations (Fig. 1). The pseudo-first-order rate constants for formation of cytosine at 100 °C are approximately proportional to the molality (*m*) of urea.

$$k \text{ (s}^{-1}\text{)} = 1.05 \times 10^{-6} m + 1.85 \times 10^{-8} m^2$$

The pH was initially adjusted to values between 5 and 9, but the small decomposition of urea to ammonium cyanate resulted in the pH rising to 9 in all the samples. The maximum yield of cytosine (Fig. 1) is approximately proportional to the urea concentration from *m*=0 to 6 (0–6 *m*), but less than this at higher concentrations. The yield of cytosine with saturated urea at 100 °C (119 *m*) is 53%. There is a drop in cytosine yield after ~30 h at 100 °C due to the hydrolysis of cytosine to uracil^{22,23}. The fall-off in maximum yield at low urea concentrations is explained by the competing reactions⁷:



The equilibrium to form the ureido-acrylonitrile is apparently unfavourable, so the cyanoacetaldehyde, which is mostly in the enolic form, is not completely converted to ureido-acrylonitrile even at the highest urea concentrations. The product shown is *cis*-ureido-acrylonitrile, but the *trans* isomer and other forms such as



would also be present. The *trans* isomer interconverts to the *cis* isomer which then forms cytosine⁵.

The temperature coefficient of the reaction was measured using 1 M urea. The Arrhenius plot (Fig. 2) gives a heat of activation of 28.2 kcal. The half-life at 25 °C is 300 years with 1 M urea and about 15 years with a saturated urea solution of 20 M.

The possibility of synthesizing 2-thiocytosine and 2-thiouracil by an analogous reaction with thiourea is attractive, because these bases occur in the transfer RNA as do a number of 5-substituted 2-thiouracils²⁴. These modified bases generally occur in the wobble position of the anticodon. Cyanoacetaldehyde reacts with thiourea to give the expected thiocytosine and thiouracil by hydrolysis. Thiourea is a potentially prebiotic compound that can be produced from NH_4SCN or by reaction of H_2S with cyanamide. However, the yield of 2-thiocytosine is only 0.05% at 100 °C with 1 M thiourea. The details of this reaction will be reported elsewhere.

After the success of the urea and thiourea reactions, we re-investigated the cyanoacetaldehyde reaction with guanidine⁷, as guanidine hydrochloride is also a very soluble compound (23 *m* at 25 °C). Yields as high as 53% of diaminopyrimidine have been obtained, compared to a maximum of 2% previously reported⁷. The rate of synthesis of diaminopyrimidine is approximately the

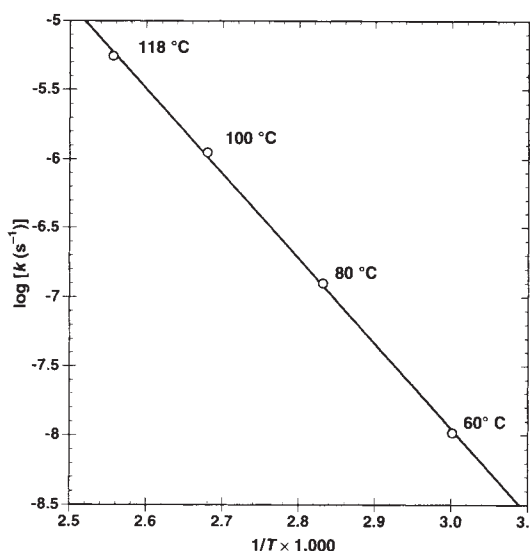


FIG. 2 Arrhenius plot of the rate constant for cytosine formation from 1×10^{-3} M cyanoacetaldehyde and 1 M urea. The line has the equation $\log k \text{ (s}^{-1}\text{)} = 10.50 - 6152/T$.

same as cytosine with urea. We will report the details of this reaction elsewhere.

The basis for the drying-lagoon model of prebiotic synthesis is that the reacting species be both non-volatile and soluble. Urea, thiourea and guanidine hydrochloride are very soluble compounds (20, 2.2 and 23 *m* respectively at 25 °C, and 120, 52 and 52 *m* at 100 °C). Cyanoacetaldehyde is essentially non-volatile. There was no loss on evaporating 100 ml of 10^{-3} M cyanoacetaldehyde solution to 10 ml. On evaporating to dryness, the cyanoacetaldehyde was largely converted to the dimeric form $\text{HOCH}=\text{C}(\text{CN})\text{CH}=\text{CHCN}$. Hydrogen cyanide is more volatile than water and so cannot be concentrated by evaporation, but it can be concentrated by freezing. This process was used in the prebiotic synthesis of purines²⁵. Similar low-temperature syntheses of pyrimidines may also be possible as the urea/water eutectic occurs at -11 °C and a urea concentration of 8 *m*.

The pyrimidine yields reported here are much higher than those of reported prebiotic purine syntheses. The relative yields on the primitive Earth are difficult to estimate, because they would depend on the abundances of the reactants in the different environments; but the pyrimidine/purine ratio may have been much higher on the primitive Earth than in the Murchison meteorite^{14,15} because of the availability of drying lagoons. These results show that pyrimidines are potential components of the first genetic material. Although the use of alternative bases is not precluded by these results, there no longer seem to be good reasons to believe that the bases used in the first informational macromolecules were radically different from those in DNA and RNA. □

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- Oró, J. *Biochem. biophys. Res. Commun.* **2**, 407–412 (1960).
- Sanchez, R. A., Ferris, J. P. & Orgel, L. E. *J. molec. Biol.* **38**, 121–128 (1968).
- Miller, S. L. & Orgel, L. E. *The Origins of Life on the Earth* (Prentice-Hall, Englewood Cliffs, NJ, 1974).
- Sanchez, R. A., Ferris, J. P. & Orgel, L. E. *Science* **154**, 784–785 (1966).
- Ferris, J. P., Sanchez, R. A. & Orgel, L. E. *J. molec. Biol.* **33**, 693–704 (1968).
- Turner, B. E. *Astrophys. J.* **163**, L35–L39 (1971).
- Ferris, J. P., Zamek, O. S., Altbuch, A. M. & Freiman, H. *J. molec. Evol.* **3**, 301–309 (1974).
- Ferris, J. P., Goldstein, G. & Beaulieu, D. J. *J. Am. chem. Soc.* **92**, 6598–6603 (1970).
- The RNA World* (eds Gesteland, R. F. & Atkins, J. F.) (Cold Spring Harbor Lab. Press, Massachusetts, 1993).
- Piccirilli, J. A., Krauch, T., Moroney, S. E. & Benner, S. A. *Nature* **343**, 33–37 (1990).
- Kolb, V. M., Dworkin, J. P. & Miller, S. L. *J. molec. Evol.* **38**, 549–557 (1994).
- Crick, F. H. C. *J. molec. Biol.* **19**, 548–555 (1966).
- Wächtershäuser, G. *Proc. natn. Acad. Sci. U.S.A.* **85**, 1134–1135 (1988).
- Stoks, P. G. & Schwartz, A. W. *Nature* **282**, 709–710 (1979).

15. Stoks, P. G. & Schwartz, A. W. *Geochim. cosmochim. Acta* **45**, 563–569 (1981).
16. Chittenden, G. J. F. & Schwartz, A. W. *Nature* **263**, 350–351 (1976).
17. Ferris, J. P., Joshi, P. C., Edelson, E. H. & Lawless, J. G. *J. molec. Evol.* **11**, 293–311 (1978).
18. Miller, S. L. *J. Am. chem. Soc.* **77**, 2351–2361 (1955).
19. Lowe, C. U., Rees, M. W. & Markham, R. *Nature* **199**, 219–222 (1963).
20. Lohrmann, R. *J. molec. Evol.* **1**, 263–269 (1972).
21. Shnidman, L. & Sunier, A. A. *J. phys. Chem.* **36**, 1232–1240 (1932).
22. Garrett, E. R. & Tsau, J. *J. pharm. Sci.* **61**, 1052–1061 (1972).
23. Shapiro, R. & Klein, R. S. *Biochemistry* **5**, 2358–2362 (1966).
24. Limbach, P. A., Crain, P. F. & McCloskey, J. A. *Nucleic Acids Res.* **22**, 2183–2196 (1994).
25. Sanchez, R. A., Ferris, J. P. & Orgel, L. E. *Science* **153**, 72–73 (1966).

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Lattice preferred orientation of olivine aggregates deformed in simple shear

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REGIONS of the Earth's upper mantle show significant seismic anisotropy due to the preferred crystallographic orientation ('lattice preferred orientation') adopted by its constituent minerals in response to deformation^{1–6}. Seismic anisotropies thus provide clues to the flow and/or stress patterns in the upper mantle, but the use of lattice preferred orientation to infer such properties from seismic data has been hampered by the lack of experimental studies relating changes in crystallographic orientation to simple-shear deformation. (Simple shear is probably the dominant mode of deformation in the upper mantle, whereas most previous experiments have focused on the effects of uniaxial compression^{7–9}.) Here we describe the results of simple-shear deformation experiments on olivine aggregates, conducted at high temperatures and pressures (~1,500 K and 300 MPa). For large strains (up to 150%), our experiments reproduce the lattice preferred orientation observed in highly deformed upper-mantle rocks, in which the olivine [100] axes lie nearly parallel to the flow direction. But for relatively small strains, the preferred orientation is rotated with respect to the flow direction, indicating that seismic anisotropy should also be sensitive to the sense of shear in the upper mantle.

Many of the important problems in solid Earth dynamics would be better understood if one could infer the flow geometry or stress orientation in the convecting mantle. Seismic anisotropy provides a potential tool for this purpose, but applications of seismic anisotropy to geodynamical problems have been difficult because of the absence of relevant experimental results. Significant lattice preferred orientation (LPO) and resultant seismic anisotropy can be produced when plastic flow occurs via dislocation creep but not when it is due to diffusion or superplastic creep^{9–11}. However, the relation between seismic anisotropy and the nature of flow in dislocation creep is not well understood. It has been established that when LPO is due solely to plastic deformation, then LPO is controlled by deformation geometry but not by stress orientation^{10,17}. However, whether LPO is related to strain^{14,15} or flow plane/flow direction^{3,10,12,13} has been controversial. When dynamic recrystallization occurs, the controlling factor for LPO is even less well understood^{7,9,11,18}. Karato^{9,11} showed that when recrystallization occurs via subgrain rotation, the LPO is similar to that due to deformation, whereas when recrystallization is due to grain-boundary migration, a completely different LPO could result, including stress-controlled LPO. In most of the previous work on seismic anisotropy, only the anisotropy projected onto the horizontal plane

has been considered^{4,6,19–21}. In these cases, differences between the models are not very important: for horizontal shear, all models predict that both the fast P-wave velocity and the polarization of the fast S-wave are parallel to the flow direction. But the distinction between these three models (strain control, flow plane/flow direction control, or stress control) becomes critical when the sense of shear, which is closely related to the dynamics of rifting (see Fig. 1), is to be inferred from seismic anisotropy²² or from LPO^{3,12,14}.

We have conducted simple-shear deformation experiments on olivine aggregates. The above three models can be distinguished by this mode of deformation but not by conventional uniaxial compression tests. Synthetic olivine aggregates were prepared by isostatically hot-pressing at 1,573 K and 300 MPa for 8 hours with added water to help grain growth²³. After hot-pressing, the samples were usually dried in a CO/CO₂ gas mixture at 1,473 K for 3 hours to remove water. In one case, the sample hot-pressed with added water was not dried to investigate the effect of water content on LPO. The total water content of the specimen after drying was ~300 p.p.m. H/Si, considerably smaller than in water-saturated olivine aggregates (~1,000 p.p.m.) prepared at otherwise similar conditions (see also ref. 24). The hot-pressed samples have very uniform grain size distributions (average grain size ~35 µm; Fig. 2a), and nearly random crystallographic orientations. Thin slices of samples (~0.5 mm thick) were cut from the hot-pressed cylinders and sandwiched between 2% thoriated tungsten pistons sawcut at 45° to the compression direction. Uniaxial compression of the pistons resulted in simple-shear deformation of the specimens. Each specimen layer was cut (per-

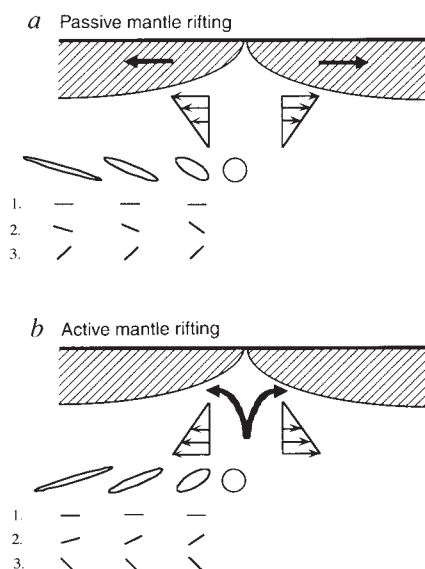


FIG. 1 Modes of rifting and corresponding flow patterns in the asthenosphere and olivine preferred orientation in a vertical plane. Hatched regions indicate the lithosphere. Flow patterns in the asthenosphere are shown by thin arrows. The evolution of the strain ellipsoid as asthenospheric materials move from the rift is also shown. Note that the sense of shear in the asthenosphere is opposite for the two modes of rifting. In the passive-mantle mode (a), rifting is supposed to occur by the action of some boundary forces or membrane stresses; the asthenospheric upwelling occurs only passively to fill the gap²⁶. In contrast, in the active-mantle mode (b), rifting is supposed to occur as a result of an active mantle upwelling current that tears the lithosphere apart. Olivine [100] axis orientations with progressive strain are shown by bars for each mode of rifting. Predictions of LPO are shown for three different models: (1) flow plane/flow direction control, (2) strain control and (3) stress control. The LPO pattern depends on the mode of rifting in strain- or stress-control model but not in flow plane/flow direction control model.

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pendicular to the layer plane) into two halves and a film of platinum ~ 20 nm thick was evaporated onto the two cut surfaces before the deformation experiment to provide a strain marker. From the rotation of the strain marker, the shear strain of the specimen was measured. To prevent sliding between the sample and pistons, grooves ~ 40 μm deep, with a spacing of 400 μm , were produced on the surfaces of the tungsten pistons. The deformation experiments were performed in a gas medium apparatus at temperatures of 1,473 or 1,573 K, a confining pressure of 300 MPa, and a shear strain rate of $(0.4\text{--}11) \times 10^{-5} \text{ s}^{-1}$. Strains up to 150% were achieved.

The microstructures of the sheared specimens were studied using both optical and electron microscopy. We found that sample strains are in good agreement with strains calculated from the piston displacement in most runs. The deformed samples show a variety of microstructural changes including grain elongation, deformation bands and grain-boundary migration (Fig. 2*b, c*). Samples deformed to shear strains higher than 50% show clear evidence of dynamic recrystallization due to both subgrain rotation and grain-boundary migration (Fig. 2*c*). The degree of recrystallization at a given strain is significantly higher at 1,573 K than at 1,473 K. Dislocation creep due mainly to the [100](010) slip system is confirmed by transmission electron microscope observations of dislocations, although in samples deformed at 1,473 K, a significant contribution from $\mathbf{b} = [001]$ dislocations was also noted.

LPO was measured using a universal stage. When recrystallization occurs, most of the specimen volume ($>80\%$) is composed of composite grains (Fig. 2*c*), indicating that the dominant mechanism of recrystallization is subgrain rotation. The LPO of these composite grains, rather than of individual subgrains, was measured in these cases. Evidence for grain-boundary migration was also seen (Fig. 2*c*). These migrating grains show distinctly different orientations from neighbouring grains^{10,11}, but their size is so small that no reliable measurements of LPO were made. The volume fraction of these grains is small ($<10\text{--}20\%$), however, and they have little effect on the bulk of LPO of the specimens. Typical pole figures are shown in Fig. 3*a, b*. The angles between [100] and [010] maxima and the flow direction are plotted against shear strain in Fig. 4. At shear strains lower than $\sim 75\%$, [100] peaks nearly follow the strain ellipsoid (ϵ_1) particularly in samples deformed at 1,473 K. At higher strains, [100] maxima become approximately parallel to the flow direction and their strengths increase with increasing strain.

Our results do not agree completely with any of the previous models for the development of LPO. On the basis of numerical modelling using a relaxed Taylor theory or the viscoplastic self-consistent theory, Wenk and Ribe and their co-workers^{14–17} showed that LPO follows the strain ellipsoid and rotates with respect to the flow plane with progressive strain. Our preferred orientation data at low strains (particularly those at 1,473 K) are roughly consistent with their prediction, but at higher strains,

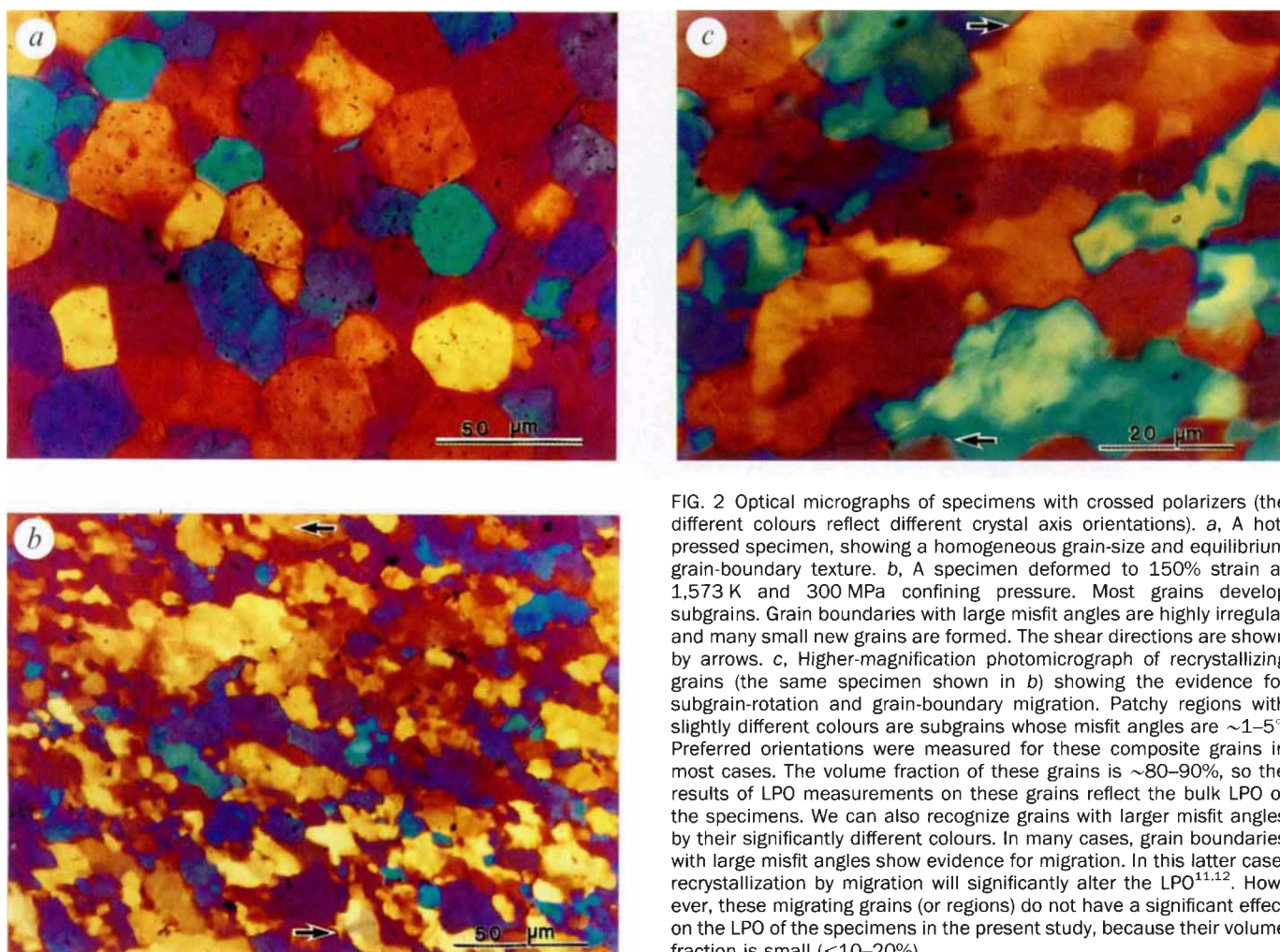


FIG. 2 Optical micrographs of specimens with crossed polarizers (the different colours reflect different crystal axis orientations). *a*, A hot-pressed specimen, showing a homogeneous grain-size and equilibrium grain-boundary texture. *b*, A specimen deformed to 150% strain at 1,573 K and 300 MPa confining pressure. Most grains develop subgrains. Grain boundaries with large misfit angles are highly irregular and many small new grains are formed. The shear directions are shown by arrows. *c*, Higher-magnification photomicrograph of recrystallizing grains (the same specimen shown in *b*) showing the evidence for subgrain-rotation and grain-boundary migration. Patchy regions with slightly different colours are subgrains whose misfit angles are $\sim 1\text{--}5^\circ$. Preferred orientations were measured for these composite grains in most cases. The volume fraction of these grains is $\sim 80\text{--}90\%$, so the results of LPO measurements on these grains reflect the bulk LPO of the specimens. We can also recognize grains with larger misfit angles by their significantly different colours. In many cases, grain boundaries with large misfit angles show evidence for migration. In this latter case, recrystallization by migration will significantly alter the LPO^{11,12}. However, these migrating grains (or regions) do not have a significant effect on the LPO of the specimens in the present study, because their volume fraction is small ($<10\text{--}20\%$).

FIG. 3 Pole figures (equal-area plot using Kamb's method²⁹) of olivine [100], [010] and [001] orientation in deformed samples and corresponding seismic anisotropy. Contours indicate the degree of preferred orientation. C is the shear plane and S indicates the foliation plane defined by the strain ellipsoid. Seismic anisotropy is calculated from the orientation of individual grains using the program by Mainprice³⁰. P-wave velocity contours (km s^{-1}), S-wave splitting(s) for a 200-km-thick layer and the map of polarization of fast S-wave orientation are shown. Seismic anisotropy in the upper mantle is caused mostly by the lattice preferred orientation of olivine, although the effects of other minerals significantly reduce the magnitude of anisotropy⁹. Therefore when upper-mantle rocks are deformed by simple shear, seismic anisotropy is similar to that shown here but has a significantly smaller amplitude. *a*, Specimen with 17% strain, deformed at 1,473 K, 300 MPa at a strain rate of $2.4 \times 10^{-5} \text{ s}^{-1}$ ($N=149$, $CI=2\sigma$), where N is the number of grains, and CI is the contour interval). *b*, Specimen with 150% strain, deformed at 1,573 K, 300 MPa at a strain rate of $1.1 \times 10^{-4} \text{ s}^{-1}$ ($N=42$, $CI=2\sigma$).

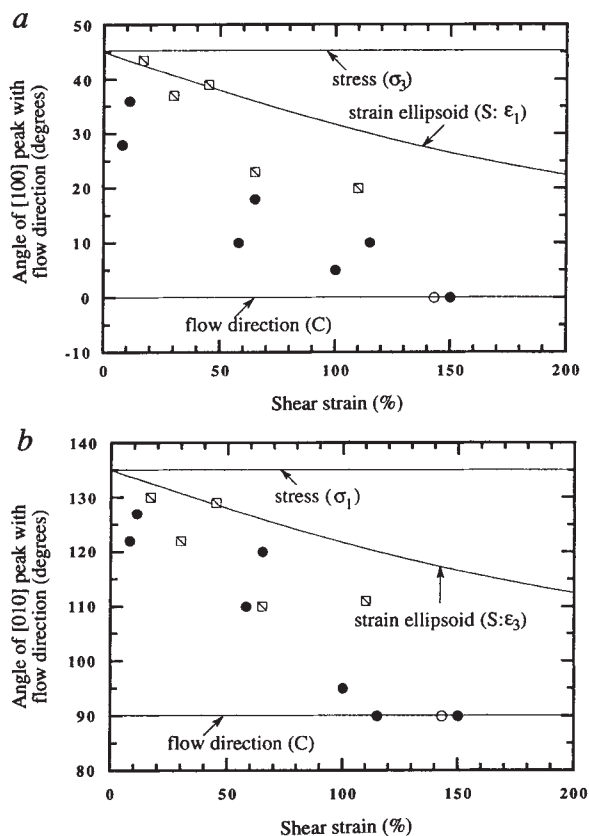
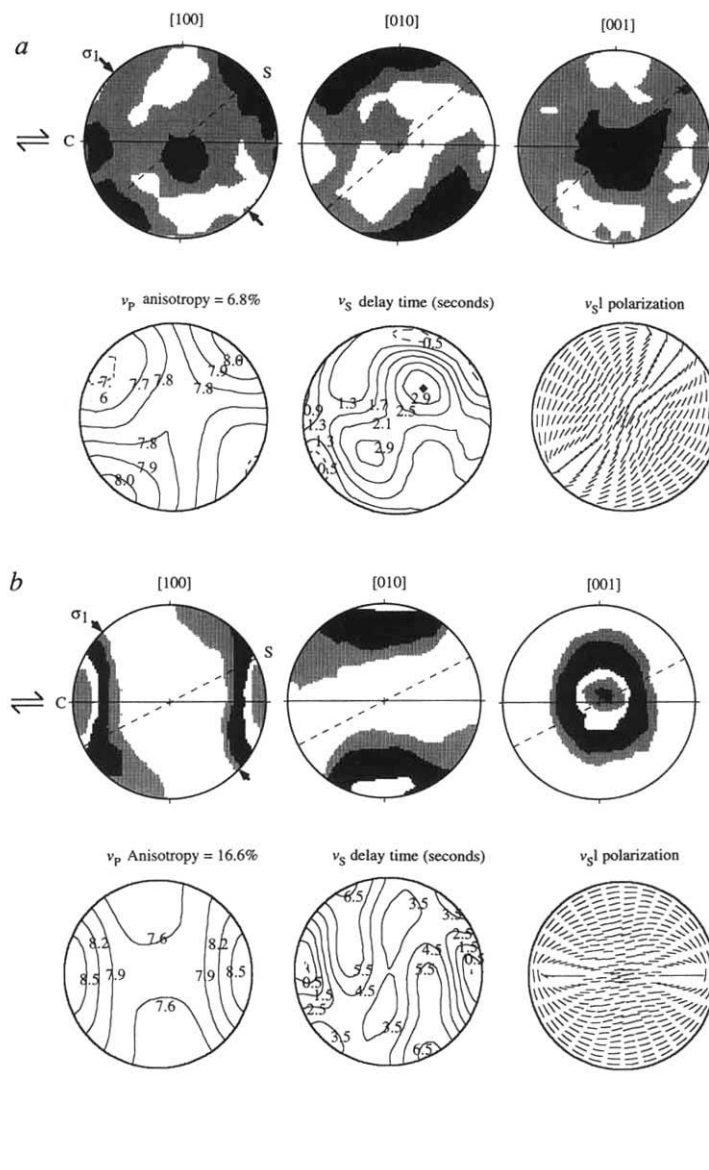


FIG. 4 The position of olivine [100] (*a*) and [010] (*b*) axis as a function of shear strain, compared with theoretical models (each data point represents the peak position based on the measurements of ~50–200 grains). The [001] axis orientation remains nearly perpendicular to the flow direction and flow plane normal throughout deformation history and is not shown. Angles of the [100] or [010] orientation maximum

are measured from the flow direction. C is the orientation of shear plane and S indicates the foliation plane. A total of 13 experiments were performed. Uncertainties in peak position are $\sim 10^\circ$ at strains $< 50\%$ and $\sim 5^\circ$ at larger strains. Filled circles show the data at 1,573 K with a small water content (< 300 p.p.m. H/Si), an open circle shows a data point at 1,573 K with water-saturated conditions, and open squares denote the data at 1,473 K with small water content (300 p.p.m. H/Si or less). The shear strain rates and shear stresses are $(0.4\text{--}11) \times 10^{-5} \text{ s}^{-1}$ and 65–239 MPa respectively. The initial densities of the samples are $(3.08\text{--}3.10) \times 10^3 \text{ kg m}^{-3}$; a few per cent uniaxial shortening strains are noticed from the change in specimen thickness after deformation. In the flow plane/flow direction control model, the peak of the [100] axis ([010] axis) is always parallel (perpendicular) to the flow direction^{3,8,12,13}. In the strain control model, the [100] ([010]) orientation is parallel to the maximum elongation direction ϵ_1 (the maximum shortening direction, ϵ_3), rotates with increasing strain, and becomes the same as in the flow plane/flow direction control model at infinite strain^{14–17}. In the stress control model, the [100] ([010]) orientation is always parallel to the minimum compression orientation, σ_3 (the maximum compression direction, σ_1)^{7,9,18}. The results at low strains (particularly at 1,473 K) agree reasonably well with the strain-controlled model, whereas the orientation becomes consistent with the flow direction/flow plane control model at large strains. A gradual change from strain control to flow direction/flow plane control is associated with an increasing degree of dynamic recrystallization.

the orientation maxima rotate at a much faster rate than the strain ellipse and the direction of the [100] peak becomes subparallel to the flow direction, consistent with the flow plane/flow direction control model^{8,10,13}. Most of our results are consistent with the previous work which showed that the LPO of grains recrystallized by subgrain rotation is similar to that of deformed grains without recrystallization^{9,11}. However, the role of migration recrystallization is not investigated in any detail in the present study.

Our results have three important implications for the interpretation of seismic anisotropy and LPO. (1) The fact that the pattern of LPO rotates with respect to the flow plane indicates that it is possible to identify the sense of shear in the asthenosphere and hence the mechanisms of rifting from seismic anisotropy (Fig. 1). Because the difference in anisotropy due to the difference in the sense of shear occurs in a vertical plane (see Fig. 1), inference of the sense of shear requires detection of anisotropy in a vertical plane, which will require sophisticated analyses of seismic records^{22,25}. (2) The use of asymmetric LPO to infer the sense of shear as proposed by Nicolas^{3,8} is justified only at large strains where dynamic recrystallization is significant. (3) We note that the present study was carried out under limited deformation conditions in which the roles of grain-boundary migration and of water are not very important. Our present results, however, seem to be applicable to the interpretations of seismic anisotropy and LPO in most of the upper mantle of the Earth for the following reasons. First, the dominant mechanism of dynamic recrystallization in the upper mantle is considered to be subgrain rotation rather than grain-boundary migration². Second, the water content in most of the oceanic upper mantle is relatively small and comparable to or even smaller than in our experiments²⁶. However, there are some indications that grain-boundary migration recrystallization might dominate in some regions of the upper mantle^{2,9} and a much higher water content in mantle minerals is expected in the wedge of mantle over the subducting lithosphere. LPO under these

conditions needs to be clarified in order to further understand geodynamics from studies of seismic anisotropy and LPO of mantle minerals^{9,11,27}. □

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- Hess, H. H. *Nature* **203**, 629–631 (1964).
- Mercier, J. C. C. in *Preferred Orientation in Deformed Metals and Rocks: An Introduction to Modern Texture Analysis* (ed. Wenk, H.-R.) 407–430 (Academic, San Diego, 1985).
- Nicolas, A. *Structure of Ophiolites and Dynamics of Oceanic Lithosphere* (Kluwer Academic, Dordrecht, 1989).
- Raitt, R. W. & Shor, G. G. *J. geophys. Res.* **74**, 3095–3109 (1969).
- Silver, P. G. & Chan, W. W. *Nature* **335**, 34–39 (1988).
- Tanimoto, T. & Anderson, D. L. *Geophys. Res. Lett.* **11**, 287–290 (1984).
- Avé Lallemant, H. G. & Carter, N. L. *Geol. Soc. Am. Bull.* **81**, 2203–2220 (1970).
- Nicolas, A., Boudier, F. & Boullier, A. M. *Am. J. Sci.* **273**, 853–876 (1973).
- Karato, S. in *High-Pressure Research in Mineral Physics* (eds Manghni, M. H. & Syono, Y.) 455–471 (Terrapub, Tokyo, 1987).
- Nicolas, A. & Christensen, N. in *Composition, Structure and Dynamics of the Lithosphere–Asthenosphere System* (eds Fuchs, K. & Froidevaux, C.) 111–123 (Am. Geophys. Union, Washington DC, 1987).
- Karato, S. *Phys. Earth planet. Inter.* **51**, 107–122 (1988).
- Nicolas, A., Bouchez, J. L., Boudier, F. & Mercier, J. C. *Tectonophysics* **12**, 55–86 (1971).
- Etchecopar, A. & Vasseur, G. *J. struct. Geol.* **9**, 705–717 (1987).
- Takeshita, T., Wenk, H.-R., Molinari, A. & Canova, G. in *Deformation Processes in Minerals, Ceramics and Rocks* (eds Barber, D. J. & Meredith, P. G.) 365–377 (Unwin Hyman, London, 1990).
- Wenk, H.-R., Bennett, K., Canova, G. R. & Molinari, A. *J. geophys. Res.* **96**, 8337–8349 (1991).
- Wenk, H.-R. & Christie, J. M. *J. struct. Geol.* **13**, 1091–1110 (1991).
- Ribe, N. M. & Yu, Y. *J. geophys. Res.* **96**, 8325–8335 (1991).
- Kunze, F. R. & Avé Lallemant, H. G. *Tectonophysics* **74**, T1–T13 (1981).
- Blackman, D. K., Orcott, J. A., Forsyth, D. W. & Kendall, J.-M. *Nature* **366**, 675–677 (1993).
- Fischer, K. M. & Yan, X. *Geophys. Res. Lett.* **21**, 5–8 (1994).
- Farra, V. & Vinnik, L. *Geophys. J. Int.* **119**, 195–218 (1994).
- Kirkwood, S. & Crampin, S. *Geophys. J. R. astr. Soc.* **64**, 463–485 (1981).
- Karato, S. *Tectonophysics* **168**, 255–273 (1989).
- Karato, S., Paterson, M. S. & Fitzgerald, J. D. *J. geophys. Res.* **86**, 8151–8176 (1986).
- Park, J. *J. geophys. Res.* **98**, 19933–19949 (1993).
- Bell, D. R. & Rossman, G. R. *Science* **255**, 1391–1397 (1992).
- Karato, S. *Proc. Japan Acad.* **71**, 61–66 (1995).
- Sengör, A. M. C. & Burke, K. *Geophys. Res. Lett.* **5**, 419–421 (1978).
- Kamb, W. B. *J. geophys. Res.* **64**, 1891–1909 (1959).
- Mainprice, D. *Comput. Geosci.* **16**, 385–393 (1990).

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Locally layered convection inferred from dynamic models of the Earth's mantle

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THE structure of convection in the mantle is still the subject of considerable debate. The now standard modelling of the convective flow as driven in a viscous mantle by density anomalies derived from seismic tomography has successfully explained the longest-wavelength (degree 2 to 8) geoid anomalies and provided important information concerning the viscosity structure of the mantle^{1–8}. With this approach, however, the predicted response of surface topography to convective stresses (the 'dynamic topography') has a typical magnitude of several kilometres, which does not conform with observations^{9–11}. A possible source of this discrepancy lies in the severe underestimation, by tomography, of density anomalies due to deflections of the boundary between the upper and lower mantle, at 660 km depth. Here we model the mantle flow implied by seismically derived density heterogeneities, using an empirical method to account for the 660-km boundary topography. The predicted dynamic (surface) topography thus obtained is significantly reduced, to values that conform with the observations; in addition, the 660-km boundary topography appears to have a strong influence on the computed mantle circulation, inducing local layering of the convective flow.

On a thousand-kilometre scale, the dominant effect in the Earth's surface topography is the deepening with age of the sea floor, due to lithospheric cooling. This plate cooling component is superimposed on the surface deformation caused by the convective flow, and should appear in surface topography predicted by mantle convection models if the tomography models used account correctly for density heterogeneities due to lithospheric thickening. Although the seismic velocity anomalies near the surface show an age dependence¹², it is generally assumed that tomography does not make proper allowance for the plate thickening. For this reason, a number of authors have computed residual depth anomalies, correcting the observed depths for age effects^{9–11}. The long-wavelength components of residual depths are expected to represent the dynamic topography. This is indeed suggested by the high correlation reported between the long-wavelength components of observed residual depth anomalies and those of the geoid and lower-mantle seismic velocity anomalies¹³. The observed dynamic topography has a magnitude of a few hundreds of metres, much less than that predicted by previous flow models^{1–8}.

This excess of surface topography is necessary to obtain a calculated geoid comparable to that observed. The corresponding density anomalies could be related to the 660-km discontinuity, which is poorly described by the density fields as inferred from tomographic models. A severe underestimation of the phase-change density anomalies results both from the poor radial resolution of current tomographic models (200 km) and from the assumption of a uniquely thermal origin during the conversion of velocity anomalies to density anomalies (the ratio of density anomaly to seismic velocity anomaly is four times larger

FIG. 1 Computed surface topography (m) (a, b) and geoid (m) (c, d) after the first step (a, c) and the second step (b, d). The fraction of surface topography transferred downwards is $C=0.5$. Density anomalies are deduced from the SH425.5 model¹⁵. The conversion factor from seismic velocity anomaly to density anomaly $(\partial \ln \rho / \partial \ln v_s)_\rho$ is 0.2 in the lower mantle and 0.4 in the upper mantle below 300 km depth. The viscosity profile is 1×10^{23} Pa s in the lower mantle, 2×10^{21} Pa s in the upper mantle below 220 km depth, and 5×10^{20} Pa s above 220 km depth.

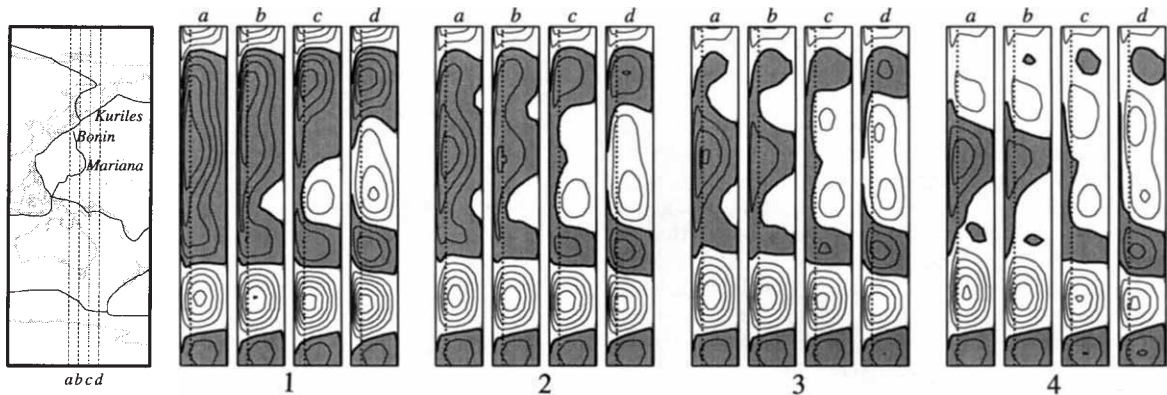
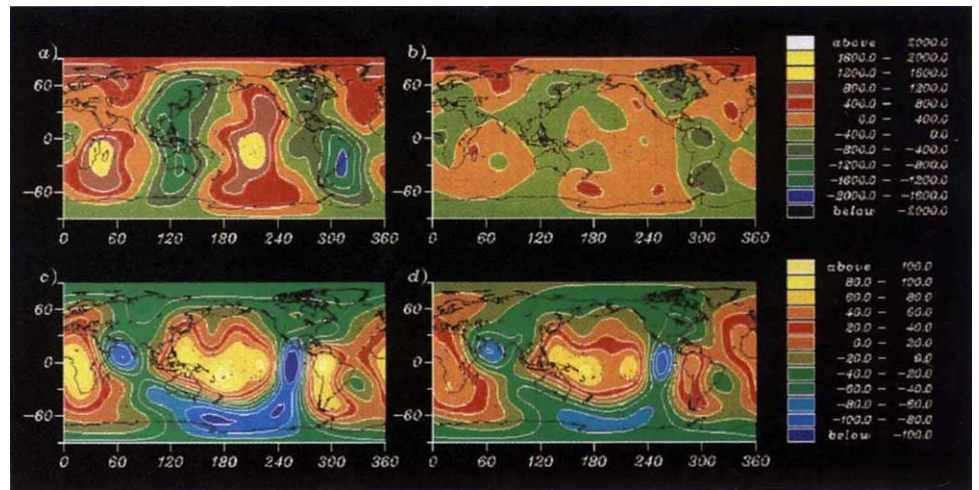


FIG. 2 Longitudinal cross-sections of the radial velocities in the mantle after the first step (panel 1) and after the second step. Contour intervals are 1 mm yr^{-1} , downwellings are shaded. The fraction of surface topography transferred downwards (C) is 0.3 (panel 2), 0.5 (panel 3) and 0.7 (panel 4). The longitudes are ($^{\circ}\text{E}$) 140 (a), 145 (b), 150 (c) and 155 (d) and correspond to the dashed lines on the map (extreme left panel). After the first step, all downwellings cross the discontinuity in subduction zones. For $C=0.5$, the 660-km depth density anomalies can locally either prevent the flow from plunging into the lower mantle (Bonin region) or let the currents cross the discontinuity (Mariana region) (Fig. 3b), in agreement with the results of seismological studies of

bending slabs. The local layering appears when the buoyancy forces related to the downward deflection of the 660-km discontinuity (lighter) balance those due to thermal anomalies (heavier). For $C=0.3$, the phase-change density anomalies are too low to induce significant changes in the convective patterns. For $C=0.7$, these density anomalies are stronger and induce a layering of the flow which is inconsistent with the observations. As these four convective structures lead to roughly the same geoid, other observational constraints such as the dynamical topography and local seismological studies are necessary to infer a reliable Earth mantle structure.

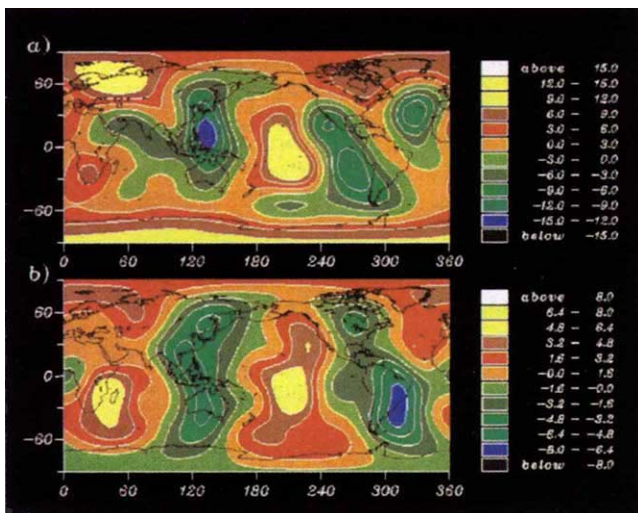


FIG. 3 a, Discontinuity topography as proposed by Shearer²² (model SH10C). b, Computed 660-km depth phase-change topography ($C=0.5$). After the first step, the excess of predicted surface topography is transferred to 660-km depth as a distribution of density anomalies, which is then expressed in terms of topography assuming the PREM¹⁸ density contrast between upper and lower mantle. The correlation between these two topographies is 84% for $l=2$ and 50 for $l=3$. However, the amplitude of the computed topography is about half that obtained by Shearer. This discrepancy is discussed in the text.

for a phase-change origin⁶). We propose an empirical method to compute the 660-km boundary deflections. Associated density anomalies tend to slow down the flow and hence reduce the computed surface topography. Indeed, surface topography and 660-km boundary topography are both deflected in the same manner by convective currents. This is due to the endothermic nature of the phase change at 660 km depth¹⁴: a downwelling produces a downward deflection of the 660-km discontinuity and vice versa. Using a now classical approach, we have developed a viscous flow model for a compressible mantle (see ref. 8 for details of the method), with lithospheric plates at the surface¹. The tomography model is that of Su and Dziewonski¹⁵. Seismic velocity anomalies in the top 300 km of the upper mantle are not considered. Indeed, most velocity anomalies in this depth range are compositional rather than thermal, as they are associated with deep continental roots¹⁶. Density anomalies due to the 660-km boundary deflections are modelled empirically through a two-step process. In the first step, the viscosity profile is determined by fitting the observed long-wavelength geoid (degree 2 to 8) and plate velocities. In terms of relative viscosity with respect to the transition zone, we use a value of 50 for the lower mantle and 0.04 for the low-viscosity zone (assumed to extend down to 220 km depth). Note that although no density anomalies are considered above 300 km depth, the viscosity of the upper mantle needs to be specified up to the surface. Conversion factors ($\partial \ln \rho / \partial \ln v_s$)_P of 0.4 and 0.2 (ρ is the density, v_s the S-wave velocity and P the pressure) have been used for the upper and lower mantle respectively¹⁷. The predicted surface topography is shown in Fig. 1a. Its magnitude is 2 km, that is, three times larger than that observed. After the first step, the model overestimates the surface deformation, indicating that the part of topography corresponding to the 1.4 km excess has another origin. We assume that a fraction C of the surface topography, computed after the first step, can be explained by density contrasts arising from the 660-km deflections. We then calculate these density contrasts so that their direct contribution to the geoid is the same as the predicted excess surface deformation, and express these density contrasts in terms of 660-km discontinuity deflections. For this purpose, the density contrast between upper and lower mantle given by the PREM model¹⁸ (390 kg m^{-3}) is used. In the second step, we recompute the geoid, the dynamic topography and the plate velocities, taking into account the empirically derived 660-km density anomalies which are now superimposed on the tomography model. Values of C ranging from 0.3 to 0.7 are used because they correspond to the fraction of predicted topography which is excessive when compared to the observed topography. The new predicted surface topography for $C=0.5$ is shown in Fig. 1b. Its magnitude is reduced to 500 m. For $C=0.3$ and 0.7, the magnitudes are respectively higher and lower than for $C=0.5$. More than 70% of the geoid variance is correctly predicted after the second step with $C=0.5$ (Fig. 1d), compared to 50% after the first step (Fig. 1c). Similarly, after the second step, the predicted poloidal plate motions are consistent with those observed¹⁹ (67% of the variance explained).

Taking the 660-km boundary density anomalies into account seems to have significant consequences for the mantle circulation. Figure 2 displays longitudinal cross-sections of the radial velocity fields at four longitudes representing the major subduction zones of the northwest Pacific. The velocity fields are shown after the first step (panel 1) and after the second step for $C=0.3$ (panel 2), 0.5 (panel 3) and 0.7 (panel 4). In panel 1, all downwelling currents cross the 660-km discontinuity whereas in panel 3, only the strongest currents penetrate the lower mantle, noticeably in the Mariana trench region. At the Bonin and South Kuriles trenches, penetration appears to be inhibited. This result agrees with seismological observations^{20,21} which have shown that slabs either penetrate the lower mantle (for example, at the Mariana trench) or are deflected at the 660-km boundary (at the Izu-Bonin trench). Recently, direct mapping of the 660-km

topography has been obtained by means of differential travel times of seismic wave phases²². This topography, shown in Fig. 3a, has a magnitude of 12 km. Phipps Morgan and Shearer⁶ used this topography along with global seismic tomography to infer the mass distribution associated with the deflection of the 660-km phase boundary, but did not investigate the question of whether these density anomalies resulted in a more realistic surface topography. Following their idea, we have used Shearer's topography²² in our flow model, along with the tomographically derived density anomalies, to infer surface topography. The total amplitude remains much too large, and in poor agreement with the observations. A tentative explanation may be sought in the lack of robustness of the 660-km topographic features in areas of poor data coverage²², but also in the fact that, as explained below, we do not take into account the effect of the 410-km seismic discontinuity. The basic motive behind the development of the present approach is that the 660-km discontinuity and surface topographies have to be correlated because of the endothermic character of the phase change: both will be deflected in the flow direction. This behaviour is observed when the currents cross the discontinuity. The higher magnitude of thermal heterogeneities in the upper mantle than in the lower mantle^{23,24} leads to a similar trend in regions where layering may occur. This similarity between surface and 660-km topographies in the case of partial layering has been re-created by numerical convection models²⁵. The interconnection between the flow patterns and the discontinuity deflections is required to obtain local layering of the flow (Fig. 2) and a significant decrease in the predicted topography. It should be noted, nevertheless, that the correlation between our computed 660-km topography and that mapped by Shearer²² is 84% for $l=2$ and 50% for $l=3$ (where l is the degree of the spherical harmonic expansion). However, its magnitude is significantly lower than that observed²². This discrepancy can be explained for the most part by two effects. First is the fact that no allowance is made for the phase change at 410 km depth. Indeed, as it is exothermic, it will be deflected upwards by a downwelling and downwards by an upwelling, and hence will be anticorrelated with both surface and 660-km depth topographies. This phase change at 410 km depth will supply a portion of the geoid, as there is a density contrast of 190 kg m^{-3} (ref. 18). Within our framework, the resulting geoid anomalies corresponding to the topographies are constant. Adding the contribution of the phase change at 410 km depth may double the magnitude of our computed 660-km topography. The second reason for underestimating the 660-km depth topography is that it could already be partially accounted for by the density anomalies deduced from tomography. To make allowance for this fact, Morgan and Shearer⁶ have proposed a correction which, within our framework, would be equivalent reducing the density contrast that relates topography to density anomalies to 280 kg m^{-3} . Applying this correction will augment our topography by 20%.

The idea of attributing the excess of surface topography to the fact that the effects of 660-km discontinuity topography have been neglected has led us to propose a new approach to the formulation of dynamic mantle convection models. The local layering of mantle convection that we derive conforms with the results of numerical convection models with endothermic phase change²⁶. Our approach makes it possible to return to a surface topography of realistic magnitude, and is consistent with independent observations, such as the bending of certain slabs and the main patterns observed in the 660-km discontinuity topography. □

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1. Ricard, Y. & Vigny, C. *J. geophys. Res.* **94**, 543–559 (1989).
2. Hager, B. H. & Richards, M. R. *Phil. Trans. R. Soc. A* **328**, 209–327 (1989).
3. Forte, A. M. & Peltier, W. R. *J. geophys. Res.* **96**, 20131–20159 (1991).
4. King, S. D. & Masters, G. *Geophys. Res. Lett.* **19**, 1551–1554 (1992).

5. Zhang, S. & Christensen, U. *Geophys. J. Int.* **114**, 531–547 (1993).
6. Phipps Morgan, J. & Shearer, P. M. *Nature* **365**, 506–511 (1993).
7. Corrieu, V., Ricard, Y. & Froidevaux, C. *Phys. Earth planet. Inter.* **84**, 3–13 (1994).
8. Thoraval, C., Machetel, P. & Cazenave, A. *Geophys. J. Int.* **117**, 566–573 (1994).
9. Colin, P. & Fleitout, L. *Geophys. Res. Lett.* **17**, 1961–1964 (1990).
10. Cazenave, A. & Thoraval, C. *Earth planet. Sci. Lett.* **122**, 207–219 (1994).
11. Kido, M. & Seno, T. *Geophys. Res. Lett.* **21**, 717–720 (1994).
12. Tanimoto, T. & Zhang, Y. S. *Geophys. Res. Lett.* **17**, 2405–2408 (1990).
13. Cazenave, A., Souriau, A. & Dominh, K. *Nature* **18**, 1257–1260 (1989).
14. Ito, E. & Katsura, T. *Geophys. Res. Lett.* **16**, 425–428 (1989).
15. Su, W. & Dziewonski, A. M. *Nature* **352**, 121–126 (1991).
16. Jordan, T. H. *Phil. Trans. R. Soc. A* **301**, 359–373 (1981).
17. Chopelas, A. & Böehler, R. *Geophys. Res. Lett.* **19**, 1347–1350 (1989).
18. Dziewonski, A. M. & Anderson, D. L. *Phys. Earth planet. Inter.* **25**, 297–356 (1981).
19. Gripp, A. E. & Gordon, R. G. *Geophys. Res. Lett.* **17**, 1109–1112 (1990).
20. Van Der Hilst, R., Engdahl, R., Spakman, W. & Nolet, G. *Nature* **353**, 37–43 (1991).
21. Fukao, Y., Obayashi, M., Inoue, H. & Nenbai, M. *J. geophys. Res.* **97**, 4809–4822 (1992).
22. Shearer, P. M. *Geophys. J. Int.* **115**, 878–904 (1993).
23. Tanimoto, T. *J. Phys. Earth* **38**, 493–509 (1990).
24. Machetel, P. in *Dynamics of Earth's Deep Interior and Earth Rotation* (eds Le Mouél, J. L., Smylie, D. E. & Herring, T.) 167–179 (Geophys. Monogr. 72, Am. Geophysical Union, Washington DC, 1993).
25. Machetel, P., Thoraval, C. & Brunet, D. *Phys. Earth planet. Inter.* **88**, 43–51 (1995).
26. Machetel, P. & Weber, P. *Nature* **350**, 55–57 (1991).

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Long-range horizontal connections and their role in cortical reorganization revealed by optical recording of cat primary visual cortex

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THE cortical 'point spread' (PS) is the area of cortex activated by a minimal visual stimulus¹. Here we use the PS to explore the functional role of lateral connectivity in normal cat primary visual cortex (V1) and its involvement in topographic reorganization of cortex following retinal lesions. We compared the distributions of PSs measured with optical recording, which reflects both spiking and subthreshold activity, with those measured with extracellular electrodes, which reveal spiking activity alone. The spiking PS represented only 5% of the area of activation shown in the optical PS, indicating that the remaining 95% was probably generated by subthreshold activation. The orientation dependence of the pattern of the subthreshold activation and its close match with orientation columns suggests that long-range horizontal connections radiating from the locus of spiking activity were responsible for the observed activation. The spike PS showed anisotropies and inhomogeneities that were related to the pattern of orientation columns and indicated distortions in the representation of visual space on the cortical surface. In the reorganized cortex the spike PS expanded, approximating the extent of the optical PS seen in normal cortex, and suggesting that reorganization was mediated by an unmasking of normally subthreshold activation to suprathreshold levels. The orientation map of the reorganized cortex showed a close match to that obtained before placing the lesion, despite the large shift in topography, supporting the idea that intrinsic horizontal connections were responsible for the remapping.

The PS in visual cortex is useful at a number of levels. It provides insight into the cortical representations of visual stimuli, is a link with the known anatomy and physiology of intracortical connections, and offers a measure of cortical plasticity. We used a combination of optical imaging and single-unit extracellular recording in cat V1 to measure the optical and spike PS in normal adult animals and in animals that had undergone

focal binocular retinal lesions. Optical recordings of the 'intrinsic signal' (that is, changes in cortical surface reflectance linked to metabolic changes resulting from local neural activity) have been used to visualize the distribution of cortical orientation columns in V1 (refs 2–4) (Fig. 1*a, b*). Images of the intrinsic signal induced in V1 by large-field grating visual stimuli (a 'global' stimulus), when colour-coded to indicate orientation preference and selectivity (a 'polar map', Fig. 1*b*), illustrate a largely continuous map of orientation, interrupted by local discontinuities ('fractures') that are associated with either rapid shifts in orientation or with areas of poor orientation selectivity^{4, 6}.

On replacing the global visual stimulus with a line segment whose length (0.5°) and location corresponded to the receptive field (RF) of a cell isolated in superficial layers of cortex, the optical PS was manifest in the resultant difference images (Fig. 1*c*). Its patchy distribution indicated a strongly orientation-selective distribution over V1, locally similar to the global orientation map (Fig. 1*a*). The polar plot of the optical PS, combining all orientations, extended over 3.2 mm along its longest axis (Fig. 1*d*). Measuring the optical PS at nine cortical sites in different hemispheres of six animals gave diameters ranging from 3.2 to 5.2 mm (mean 3.88 ± 0.62 mm). The diameter was independent of retinotopic location, despite differences in magnification factor associated with different eccentricities relative to the area centralis.

The first striking feature of the optical PS was its large size, in terms both of its extent in the visual field compared to RF diameters and of its size relative to that of the spiking PS. The optical PS shown in the example illustrated (Figs 1–3) represented an extent of 4° in the vertical dimension in visual space (Fig. 2*a*), which was much larger than RF diameters of superficial layer cells in this part of V1 (0.3 to 1°). Similar results were obtained in the other eight cases, with the optical PS representing visuotopic extents ranging from 2.25° (near the area centralis, where RF diameters were measured at ~0.25°) to 6° (at 7° eccentricity, where RF diameters were measured at ~0.75°). The RFs obtained from opposite sides of the optical PS were widely separated, both from each other and from the stimulus used to generate the PS. An RF profile mapped with a computer-generated grid of stimuli gave RF dimensions comparable to those seen with hand mapping (3/4°; Fig. 2*b*, left), corroborating this lack of overlap. An interaction RF profile of the same neuron (which provides a measure of the modulatory influences surrounding the RF by using paired conditioning and test stimuli⁷; Fig. 2*b*, right), however, showed an inhibitory moat surrounding the excitatory centre which extended out ~4°, comparable to the visuotopic extent of the optical PS. The intrinsic optical signal, because of its association with metabolic activity, could reflect inhibitory as well as excitatory activity: it is known that horizontal connections contact inhibitory (smooth stellate) interneurons as well as making direct connections with excitatory (pyramidal) neurons, and it has been shown in cortical slices that the activation of horizontal inputs can have a predominantly inhibitory effect. It is therefore plausible that the mechanisms generating subthreshold modulating influences in the RF surround may be closely linked to the cortical interactions revealed by the optical PS.

The spike PS was measured with a denser set of electrode penetrations in order to determine the maximal area of cortex containing cells whose RFs overlapped the 'point' stimulus, which allowed for a more precise comparison with the size of the optical PS. Consecutive penetrations were placed in a grid-like pattern with a separation of ~150 µm. For each penetration, several RF measurements were made within the superficial layers, to measure both RF size and scatter, and RF overlap was determined within and across penetrations. The spike PS extended 400 µm anteroposterior and 1 mm mediolateral in cortex (sites 3, 4, 5, 11 and 12 in Fig. 2*c* have RFs overlapping with the point stimulus). This area was somewhat smaller than that seen in primate studies, which indicate that a minimum

distance of two hypercolumns (~ 1.5 mm) is required for cells to have non-overlapping RFs⁸. The anisotropy of spike PS could be related to its registration with orientation and ocular dominance columns^{9,10}. Closely spaced electrode recordings in a second case, with an optical PS diameter of 3.5 mm, gave a spike PS of $500 \mu\text{m} \times 1$ mm; in the other seven cases, sparser electrode recordings (on a grid of $500 \mu\text{m}$) consistently gave an outer limit of $1 \text{ mm} \times 1$ mm to the spike PS. Spike PSs measured at ten different cortical sites in an additional five animals ranged from 400 to $1,100 \mu\text{m}$ in diameter (mean $740 \pm 170 \mu\text{m}$). A comparison of these numbers with the diameters of the optical PSs revealed that 95% of the optical PS area lay outside the area of the spike PS and probably reflected subthreshold activation of the targets of the intracortical projections of cells stimulated within the spike PS.

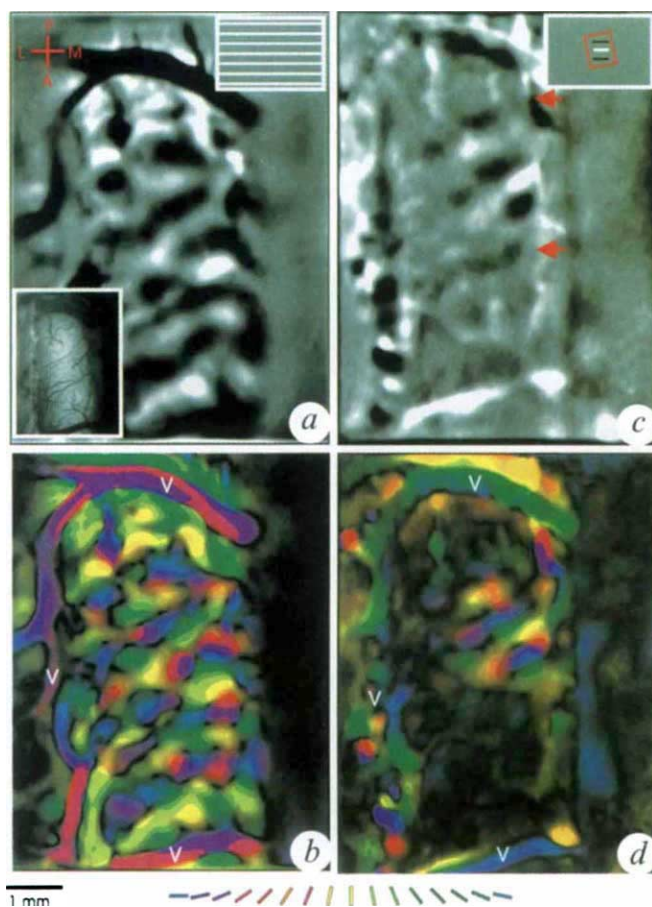
The second striking feature of the optical PS was the distribution of orientation preferences over its area, which closely matched that of the global orientation map (Figs 1*b, d* and 3).

FIG. 1 Orientation columns and optical PS in adult cat primary visual cortex (V1), based on optical images of intrinsic signals, obtained using a full-field grating (*a, b*) or small moving bar (*c, d*). Left inset of *a* is a surface view of the exposed portion of V1. Parts *a–d* and the optical maps in Figs 2 and 3 are of this segment of V1, and are shown in this orientation. *a*, Image of intrinsic cortical signal stimulated with vertical grating subtracted from intrinsic signal stimulated by a horizontal grating. Dark patches correspond to cortical regions preferentially stimulated by the horizontal grating and white patches those preferentially stimulated by the vertical grating. Right inset indicates stimulus pattern of full field gratings. *b*, Polar plot showing computed orientation preference and strength of optical activity generated by the global stimulus. Orientation-preference is colour-coded as follows: the 0 – 180° range is binned into 16 equal divisions at intervals of 11.25° , as indicated by the oriented coloured line segments at the bottom. Thus the first colour (blue) indicates orientations ranging from 0 to 11.25° , the fifth (red) from 45 to 56.25° , the ninth (yellow) from 90 to 101.25° and the 13th (green) from 135 to 146.25° . The brighter regions mark areas of high orientation signal (vector) strength, which implies a strong optical signal combined with high orientation selectivity. Darker regions mark areas of low orientation signal strength; large dark areas mark regions of weak optical signal or noise; dark points or lines within a bright region indicate sites of poor orientation selectivity or rapid changes in orientation preference ('fractures' within the orientation map). The same key is used in Figs 1–4. V, vascular artefact. *c*, Optical point-spread (PS, region bracketed by the two arrowheads) with pattern of activation elicited by vertical bar subtracted from that elicited by a horizontal bar, showing its considerable size. Inset: stimulus (white bar) moved inside region delimited by black bars, contained within an individual RF (red outline). *d*, Polar plot of optical PS. Optical image is strong and highly orientation selective over the same region of cortex bracketed in *c*, and weak over the rest of the exposed V1.

METHODS. Adult cats were anaesthetized, paralysed and prepared for electrical and optical recording from V1²², exposing the segment of V1 shown in the lower inset. To eliminate any residual eye movement, eyes were fixed relative to the stereotaxic apparatus using a ring glued to the sclera. Stability of eye position was confirmed by monitoring RF positions through each experiment. The brain surface was illuminated with light at 610 nm (interference filter, Oriel, 30 nm). Drifting gratings were presented at each of eight orientations spanning 180° , filling the animal's visual space. For each orientation, video images of the brain surface were digitized and accumulated at video rates (Imager 2001 system from Optical Imaging Inc., using Bischke CCD-6012P camera and Matrox IM-640 imaging board. Imager was controlled by VDAQ software and images were analysed using TVMIX as well as custom software.) Images obtained with a grating at one orientation were then subtracted from images with the orthogonal grating to correct for non-specific stimulus-driven changes in cortical reflectivity (see *a*, for example, 0 – 90°). The set of all orientation difference images was combined vectorially, pixel by pixel, to calculate the strength and preferred orientation of the resultant optical signal which is shown in polar plots. Orientation preference is encoded by a false colour key; the brightness of the colour is proportional to the strength of the resultant orientation signal⁴. The regions of spurious orientation signal outside the cortex are thus eliminated, although vascular artefacts due to large blood

This could be most clearly seen in a pixel-by-pixel map of orientation differences between the optical PS and the global map (Fig. 3*b, c*), where the difference was close to 0° over the area of the optical PS but not in the surrounding region. The match was consistent with the pattern of activation expected to be generated by long-range horizontal connections^{11–14} radiating out from neurons contained in the spike PS. These connections are known to mediate interactions between columns of similar orientation preference^{15,16}, although feedback connections could also have contributed to the spread of activation. The match also served as a control, indicating that none of the spread of the optical PS could be attributed to scatter of light in the cortex or to lateral spread of activity-linked blood flow signal tied only to the spiking activity.

Thus the hypothesis that the optical PS reflected subthreshold activity in the targets of the intracortical projections was supported by several findings: the comparison of the sizes of optical and spike PSs, the visuotopic extent of the optical PS relative



vessels that show uncontrollable dilation still remain. A single neuron, whose receptive field (RF) is indicated by the red rectangle in the inset (*c*) was then isolated in the superficial layers of V1. A small bar, $1/2^\circ \times 1/10^\circ$, sweeping over a distance of $1/2^\circ$, was positioned in the RF. Although this is not, strictly speaking, a point stimulus, reducing the size of the bar was found not to reduce the size of the resulting PS. The bar was presented at the same orientations used in the grating stimulus, and optical images of the stimulated intrinsic cortical activity were acquired at each orientation. These individual images were similarly processed to give difference images such as the 0 – 90° difference image shown in *c*, analogous to the image in *a*. The individual orientation difference images were then combined to give the resultant orientation preference and strength, represented in the polar plot of the point spread (*d*). The image appeared within 5 min; further averaging for periods up to one hour improved its contrast without changing its extent.

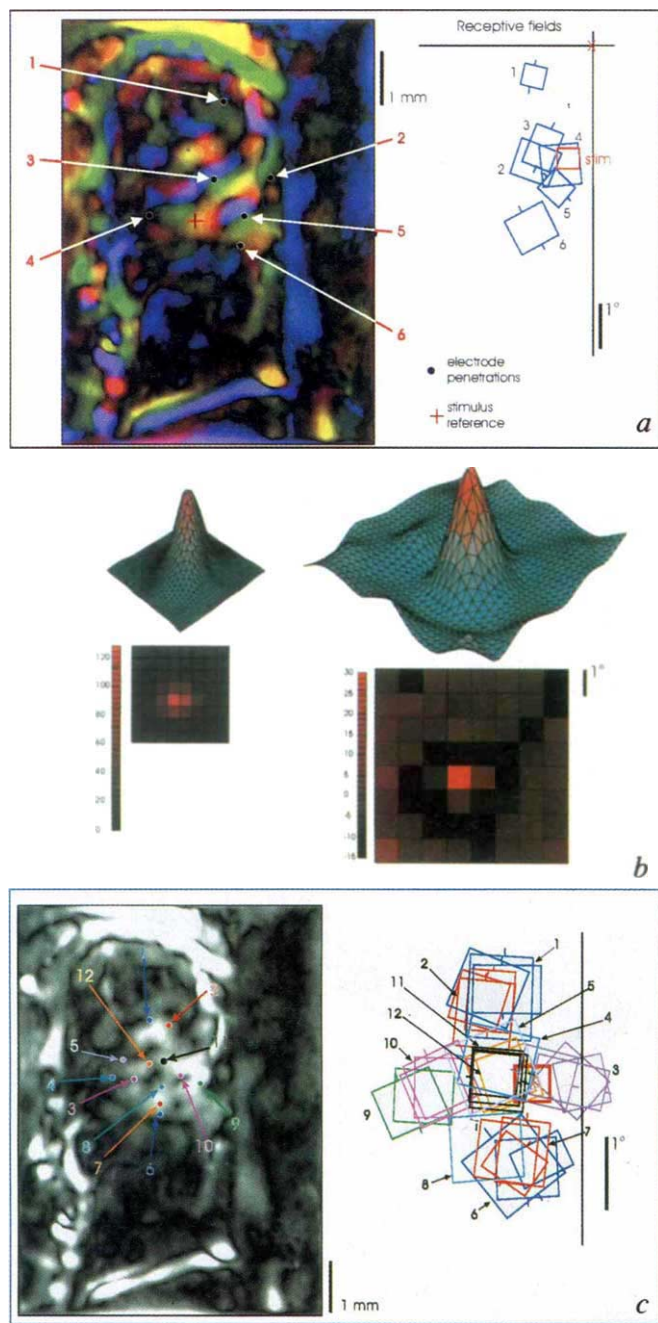


FIG. 2 Relationship between optical PS, spike PS and RF sizes. *a*, Optical PS shown in Fig. 1. Electrode recording sites marking the perimeter of the optical PS (extending 3.2 mm in cortex), are shown in relation to the recording site used for positioning the PS stimulus. The corresponding RFs are shown on the right in relation to the location and traverse of the PS stimulus ('stim'). *b*, RF profiles for the neuron recorded at position 2 in *a*, using computer-generated bar stimuli presented on a grid spanning the RF. Left profile was obtained with a single test stimulus activating the neuron from different visual field locations, forming the vertices of an 8×8 grid spanning the RF. For the right profile, the test stimulus was similarly presented at the vertices of a larger 8×8 grid while simultaneously stimulating the neuron with a weak conditioning stimulus positioned over the centre of the RF in order to bring out both inhibitory and excitatory interactions between the conditioning and test stimuli. On the left, the 3-dimensional profile shows the neuron spike rate (above spontaneous) measured as a function of the position of the test stimulus. On the right, the 3-dimensional profile shows the neuron spike rate as a function of the position of the test stimulus, after subtracting the spike rate generated by the conditioning stimulus alone. Note the inhibitory surround of the RF. The neuron spike rate measured as a function of test stimulus grid position is shown below each profile, with spike rates colour-coded as shown. Note that on the right, spike rates for (test + conditioning stimuli) are given relative to the rates for conditioning stimulus alone. Hence the negative numbers for the inhibitory surround of the RF. Grid size is marked in degrees in visuotopic space, and shows the span of the grid of test stimuli. *c*, Detailed map of RF scatter and spread. Numbered positions on the left mark the positions of electrode penetrations, displayed in relation to the strength of the optical PS orientation map. Neurons were isolated at various depths up to 600 μm in each penetration. The corresponding hand-mapped RF outlines are shown on the right, with the different RFs measured at each penetration site colour-coded for that site.

METHODS. *a*, Electrode penetrations were first made around the perimeter of the optical PS. The RFs at each site were located on a tangent screen using a hand-held visual stimulus by listening to the output of the recorded neuron through an audio monitor. The measured RFs are shown on the right, in relation to the location and limits of movement of the stimulus used for generating the PS. *b*, Individual RF profiles were then measured using automated stimulus-generation and spike-counting programs. For the left RF profile, the stimulus consisted of a single test bar, $1/2^\circ \times 1/10^\circ$ in size and optimally oriented for the neuron, making $1/2^\circ$ sweeps, with sweep centres jumping pseudo-randomly over an array of points. These sweep centres formed a square 8×8 grid with $1/2^\circ$ spacing, at the neuron's optimal orientation and positioned over the hand-mapped RF. Stimulus-driven spikes were recorded automatically and binned over each sweep of the bar. Spike rates at each grid position are displayed with the indicated colour coding, both as an 8×8 grid and as a spline-fitted 3-dimensional profile. The right (interaction) RF profile was obtained with a pair of bar stimuli: the test stimulus, also $1/2^\circ \times 1/10^\circ$ in size with a $1/2^\circ$ sweep, was moved over a similar grid of sweep centres, at a 1° spacing, while a second bar, the 'conditioning stimulus', $1/4^\circ \times 1/10^\circ$ in size with a $1/2^\circ$ sweep, was centred over the hand-mapped RF to provide steady and continuous stimulation. Bar contrasts were chosen to ensure that the neuron remained well below saturation. Spikes were collected for each sweep of the test + conditioning bars, as well as for sweeps of the conditioning stimulus alone with no test stimulus. The average spike rate measured for the conditioning stimulus alone was subtracted from the spike rate measured at each grid position of the test (with conditioner) to give the interaction RF grid and profile on the right, also colour-coded as indicated.

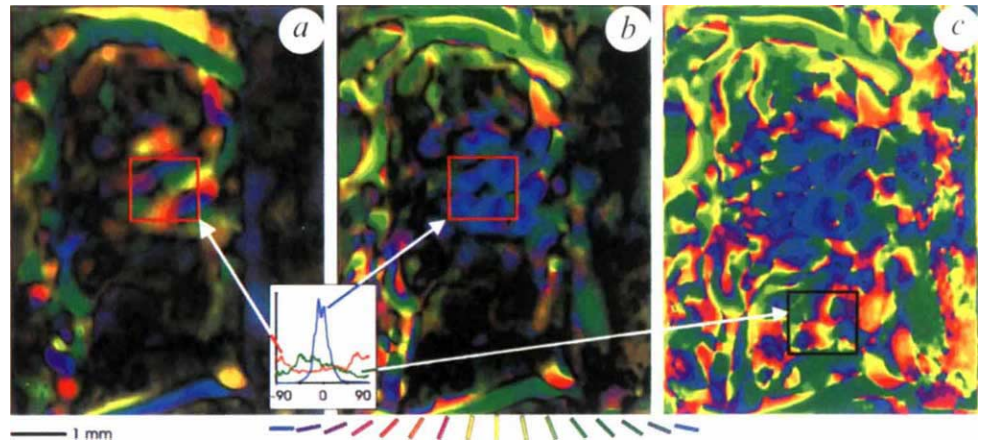
to the sizes of neuronal RFs, and the match of orientation in the optical PS with a global map. The use of optical recording based on signals generated by voltage-sensitive dyes had previously shown unexpectedly large areas of activation, which could be attributed to subthreshold influences¹⁷. By combining measures of spiking PSs and optical PSs based on intrinsic signals, the pattern of subthreshold activation can be directly visualized and its functional consequences explored.

The visuotopic map in Fig. 2c was distinguished by its inhomogeneity. Neighbouring penetrations in some areas showed considerable overlap, whereas these in other areas showed rapid shifts in RF position, giving the map a 'tiled' appearance. This inhomogeneity in cortical magnification factor

correlated with the regions of high and low orientation signal strength seen in the optical images, such that sites with overlapping RFs (sites 1 and 2; sites 3, 4, 5, 11 and 12; sites 6, 7 and 8) clustered within a single contiguous region of high orientation signal strength and of local continuity of orientation. On the other hand, the 'fractures' of low net orientation strength, possibly associated with rapid changes in orientation and/or poor orientation selectivity, were also associated with the rapid shifts in RF position. A relationship between orientation domains and topographic order was suggested by a topological model¹⁸, although the specific nature of the predicted relationship is at variance with our findings.

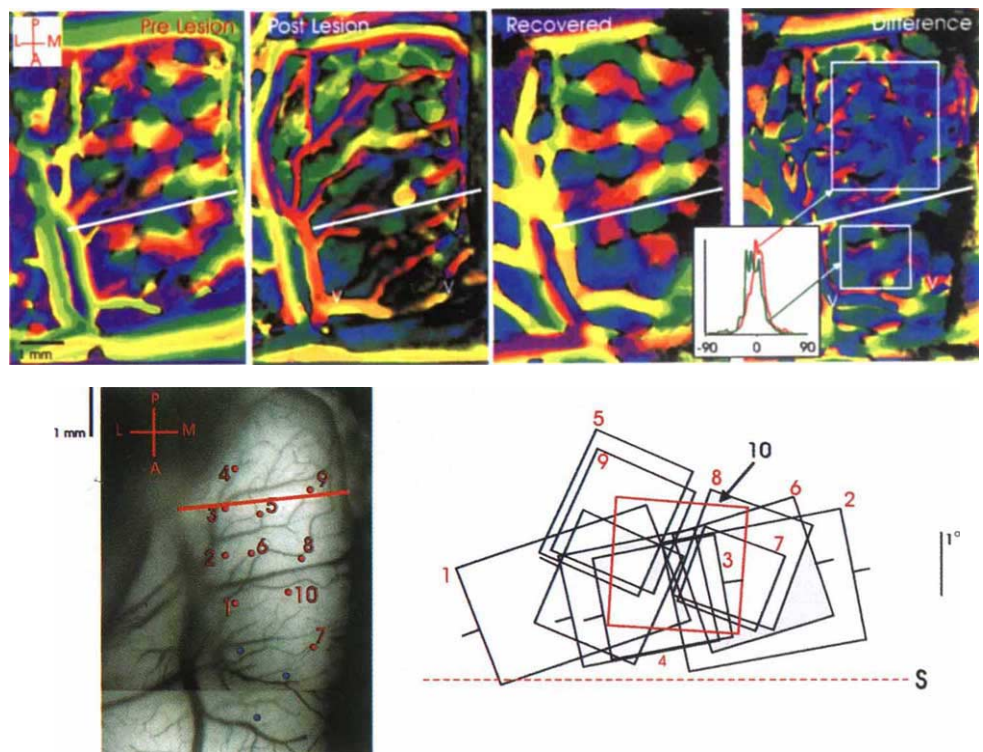
The spread of subthreshold cortical activation reflected in the optical PS was used to monitor cortical reorganization after

FIG. 3 The distribution of orientations in the optical PS matches the distribution in the global orientation map. *a*, Polar map of optical PS, showing the region used for calculating histograms of orientation distribution. *b*, Orientation difference map. Orientation preferences calculated for the global map were subtracted, pixel by pixel, from the orientation preferences for the optical PS; brightness map same as that for the optical PS. The orientation differences are encoded by the same false colour scheme as the orientations, where blue corresponds to 0° . *c*, Orientation difference map: as *b*, but encoded for the angle only, without modulating the brightness to show orientation selectivity or optical signal strength. Orientation difference was close to 0° only over the region with a strong optical PS signal, while it showed the full scatter of spurious angle-difference values where the optical PS was weak or non-existent. Inset: histograms of orientation (red, from PS) and orientation difference (blue, from region of strong PS signal; green, outside PS



region). PS orientation differences, peak centred at 0° , full width at half maximum FWHM = 30° . Histograms plot the number of pixels at each orientation, as sampled in the V1 region enclosed by the boxes shown in each case. All three boxes have the same dimensions; for *a* and *b*, the boxes are also in the same location.

FIG. 4 Upper panels, optical maps taken before and after retinal lesions show an invariance in the pattern of orientation columns, suggesting involvement of long-range horizontal connections in the reorganization. All optical images are of orientation maps elicited by full field gratings (V1, right hemisphere) and are colour-coded for orientation preference and orientation signal strength like earlier polar plots. The upper edge of the initial cortical scotoma (taken from the position of the lesion boundary on the initial cortical map) is shown on all four images (white line). In the 'post-lesion' map, a residual optical image of orientation extended into the cortical scotoma, despite the absence of visually driven activity. The pattern of orientation was similar to that seen pre-lesion (in the region immediately below the scotoma boundary; V, vascular artefact). In the 'recovered' cortex, visually driven activity had returned, with the RFs shifted to positions outside the retinal lesion. The optical orientation map stayed roughly unchanged from the pre-lesion state, despite reorganization of RF input. This is highlighted in the (recovered cortex – pre-lesion) 'Difference' map. The inset histograms of orientation difference from regions of reorganized cortex (green) and normal cortex (red) are both centred around 0° (reorganized: median, 6° ; FWHM = 28° ; normal: median, 4° , FWHM = 24°). The white rectangles indicate the regions of the animal used for calculating the histograms. Lower panels, spike point spread expanded after cortical reorganization to dimensions of earlier subthreshold region (different animal from the upper set). Left, sites for extracellular electrode recordings made from cortex of animal five months after matched binocular retinal lesions, shown in relation to the vasculature, V1, surface view. The recording sites shown in red include both part of the original cortical scotoma (region lying below the red line) and the area of normal cortex immediately adjacent; those in blue did not show visually driven spiking activity. Right, Corresponding RF positions. Because of the overlap of all the recorded RFs with each other, a test stimulus positioned within the RF of recording site 10 would drive neurons to spike over the entire cortical region that contained recording sites



1 to 10, producing a spike PS of dimensions 4.5×2.5 mm. S, upper boundary of retinal lesion.

METHODS. Adult cats were surgically prepared for optical imaging. After making global orientation map ('Pre Lesion') and electrophysiological recordings, an ophthalmological infrared laser (IRIS Instruments: pulses 800 mW $\times$ 800 ms each) was used to ablate visuotopically selected, matching regions of both retinæ. The boundaries of the scotoma were verified in cortex with electrode recordings. A fresh global orientation map was obtained immediately following retinal lesion ('Post Lesion'). After a recovery period of five months electrophysiological recordings were repeated on the same region of cortex, confirming that the cortex had recovered visually driven spike activity over a region extending over 3 mm past each of the original borders of the cortical scotoma. An optical map of this region ('Recovered') was subtracted pixel by pixel from the pre-lesion map, with the help of vascular landmarks, to give the 'Difference' map.

creating binocular retinal lesions (Fig. 4). Immediately following a lesion, a degree of optically recorded activity could be seen beyond the region of spiking activity, and within the cortical projection of the lesion boundaries (the 'cortical scotoma'). The extension of optically recorded activity inside the cortical scotoma was consistent with the subthreshold extent of the optical PS seen in normal cortex. After five months, the cortical scotoma had shrunk (Fig. 4), with the reorganized cortex receiving input from superimposed RFs on one or the other side of the lesion^{19–22}. Notably, even though the retinotopic inputs to the reorganized cortex had moved to a very different part of visual space, the orientation map in this region of cortex roughly matched the map in the same region before the lesion. This match could be quantified by the histogram of the pixel-by-pixel difference in orientation, which was sharply peaked around 0° and not discernibly different, in position and width, from the corresponding histogram from normal cortex outside the reorganized region. Similar results were obtained from three hemispheres (two animals), with the corresponding difference histograms centred near 0° (ranging from 0° to 15°) and having bandwidths ranging from 24 to 33° (full width at half maximum). This match was consistent with the reorganization being mediated by the horizontal connections^{22–24}. The overlapping RFs recorded from the reorganized region as well as the adjacent normal cortex, an area covering over 4 mm, also implied that this entire region could now be driven to suprathreshold levels by a small visual stimulus positioned in the area of RF overlap. In reorganized V1, therefore, the spike PS now extended over the same area covered by the optical PS in normal cortex.

A combination of electrophysiological recordings and optical imaging revealed the considerable extent and specificity of lateral cortical interactions, mirroring the known extent and connectivity of long-range horizontal collaterals in V1. The same technique allowed us to visualize the progress of reorganization in cortex following a restricted binocular lesion. The correspondence in the orientation maps of prelesion and post-reorganization cortex, and the increase in the area of the spiking PS, implied that the process of reorganization depended on a strengthening of the existing scaffolding of horizontal connections. The cortical processes revealed by combining optical and spike PS measurements allow us to explore their functional consequences and their posited involvement in manifestations of visual spatial integration. These may involve cortical dynamics and lead to perceptual consequences such as normalization, perceptual constancies, perceptual fill-in and perceptual learning. □

Distinct aspects of neuronal differentiation encoded by frequency of spontaneous Ca²⁺ transients

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STIMULATION of transient increases in intracellular calcium (Ca²⁺) activates protein kinases^{1–3}, regulates transcription^{4–9} and influences motility and morphology^{10–12}. Developing neurons generate spontaneous Ca_i²⁺ transients, but their role in directing neuronal differentiation and the way in which they encode information are unknown. Here we image Ca²⁺ in spinal neurons throughout an extended period of early development, and find that two types of spontaneous events, spikes and waves, are expressed at distinct frequencies. Neuronal differentiation is altered when they are eliminated by preventing Ca²⁺ influx. Reimposing different frequency patterns of Ca²⁺ elevation demonstrates that natural spike activity is sufficient to promote normal neurotransmitter expression and channel maturation, whereas wave activity is sufficient to regulate neurite extension. Suppression of spontaneous Ca²⁺ elevations by BAPTA loaded intracellularly indicates that they are also necessary for differentiation. Ca²⁺ transients appear to encode information in their frequency, like action potentials, although they are 10⁴ times longer in duration and less frequent, and implement an intrinsic development programme.

Long-term imaging of cultured *Xenopus* embryonic neurons (Fig. 1a) reveals patterns of Ca_i²⁺ elevation through the Ca²⁺-sensitive period, during which removal of Ca²⁺ inhibits maturation of the rate of activation of delayed rectifier potassium current (*I*_{Kv}) and the normal appearance of GABA (γ-aminobutyric acid), while potentiating outgrowth of processes^{13–17}. Over a period of 18 h, spike frequency is initially low and rises to 3 per h at 5–10 h *in vitro*, declining to 1 per h subsequently (Fig. 1b). In contrast, wave frequency is constant at ~8 per h in growth cones, after neurite extension is initiated, and varies around 1 per h in somata.

To investigate the functions of spontaneous Ca²⁺ elevations in regulating different aspects of development, we first eliminated them in Ca²⁺-free medium¹⁸. Their ability to direct neuronal differentiation was then determined by stimulation of transients to mimic spike and wave frequencies occurring naturally during the 12-h periods of greatest activity. Ca²⁺ transients were generated by 20–30 s pulses of a solution containing 100 mM KCl and 10 mM CaCl₂, depolarizing neurons and stimulating Ca²⁺ influx throughout cells. During interpulse intervals, cultures were constantly perfused with Ca²⁺-free medium. Stimulated Ca²⁺ transients examined with Fluo-3 mimic spikes in amplitude in the soma but are longer in duration at half-peak (Fig. 2a). They also resemble waves at growth cones in both amplitude and duration (Fig. 3a).

Stimulating Ca²⁺ elevations to mimic spike frequency allows neuronal differentiation to proceed normally. The time course of activation of *I*_{Kv} in stimulated cultures is not significantly different from that in Ca²⁺-containing cultures (*P*=0.47), and is significantly faster than that in Ca²⁺-free cultures (*P*<0.001; Fig. 2b). Furthermore, 45±3% of neurons in stimulated cultures exhibit GABA-immunoreactivity at 1 day, similar to 43±3% of neurons in Ca²⁺-containing cultures (*P*=0.63), and significantly higher than 12±2% of neurons in Ca²⁺-free cultures (*P*<0.001; Fig. 2c).

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- McIlwain, J. T. *J. Neurophys.* **38**, 219–230 (1975).
- Grinvald, A., Lieke, E. E., Frostig, R. D., Gilbert, C. D. & Wiesel, T. N. *Nature* **324**, 361–364 (1986).
- Frostig, R. D., Lieke, E. E., Ts'o, D. Y. & Grinvald, A. *Proc. natn. Acad. Sci. U.S.A.* **87**, 6082–6086 (1990).
- Ts'o, D. Y., Frostig, R. D., Lieke, E. E. & Grinvald, A. *Science* **249**, 417–420 (1990).
- Blasdel, G. G. & Salama, G. *Nature* **321**, 579–585 (1986).
- Bonhoeffer, T. & Grinvald, A. *Nature* **353**, 429–431 (1991).
- Bishop, P. O., Coombs, J. S. & Henry, G. H. *J. Physiol., Lond.* **219**, 659–687 (1971).
- Hubel, D. H. & Wiesel, T. N. *J. comp. Neurol.* **158**, 295–306 (1974).
- Hubel, D. H. & Wiesel, T. N. *Proc. R. Soc. B* **198**, 1–59 (1977).
- Tootell, R. B. H., Silverman, S., Switkes, E. & DeValois, R. L. *Science* **218**, 902–904 (1982).
- Gilbert, C. D. & Wiesel, T. N. *Nature* **280**, 120–125 (1979).
- Gilbert, C. D. & Wiesel, T. N. *J. Neurosci.* **3**, 1116–1133 (1983).
- Rockland, K. S. & Lund, J. S. *J. comp. Neurol.* **216**, 303–318 (1983).
- Martin, K. A. C. & Whitteridge, D. *J. Physiol., Lond.* **353**, 463–504 (1984).
- Ts'o, D. Y., Gilbert, C. D. & Wiesel, T. N. *J. Neurosci.* **8**, 1160–1170 (1986).
- Gilbert, C. D. & Wiesel, T. N. *J. Neurosci.* **9**, 2432–2442 (1989).
- Grinvald, A., Lieke, E. E., Frostig, R. D. & Hildesheim, R. *J. Neurosci.* **14**, 2545–2568 (1994).
- Durbin, R. & Mitchinson, G. *Nature* **343**, 644–647 (1990).
- Gilbert, C. D., Hirsch, J. A. & Wiesel, T. N. *Cold Spring Harb. Symp. quant. Biol.* **55**, 663–677 (1990).
- Kaas, J. H. et al. *Science* **248**, 229–231 (1990).
- Heinen, S. J. & Skavenski, A. A. *Expl Brain Res.* **83**, 670–674 (1991).
- Gilbert, C. D. & Wiesel, T. N. *Nature* **356**, 150–152 (1992).
- Darian-Smith, C. & Gilbert, C. D. *Nature* **368**, 737–740 (1994).
- Darian-Smith, C. & Gilbert, C. D. *J. Neurosci.* **15**, 1631–1647 (1995).

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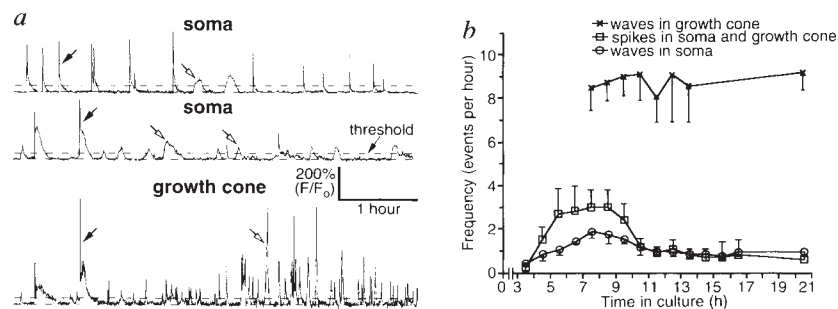


FIG. 1 Patterns of spontaneous neuronal Ca^{2+} spikes and waves over the period in which differentiation is altered by removal of extracellular Ca^{2+} . Sequences of fluorescence images of cells loaded with Fluo-3-AM were acquired by confocal microscopy. *a*, Spikes (filled arrows) and longer waves (open arrows) recorded from somata of two neurons during a 5-h period from 7–12 h in culture, (top). Spikes and waves recorded from a growth cone of the second neuron during the same period (bottom). Waves are generated at higher frequencies and are briefer in growth cones than in somata, although longer than spikes (33 ± 4 s versus 9 ± 1 s; mean $\pm$ s.e.m., $n > 20$). Previous analysis showed that spikes are propagated throughout neurons, associated with Ca^{2+} action potentials, and eliminated by calcium-channel blockers, whereas waves propagate decrementally and only large waves in the soma are detected at growth cones¹⁸. Waves in both soma and growth cones involve an unidentified Ca^{2+} pathway and are not affected by blocking spikes. *b*, The incidence of spikes is temporally regulated and is highest between 5 and 10 h in culture. In contrast, the incidence of waves is spatially regulated, is higher in growth cones than in cell bodies, and is constant in growth cones during the period examined. During the period from 7–12 h in culture, 90% of neurons exhibited spontaneous Ca^{2+} transients. Seventy per cent produced spikes and 75% produced waves; 55% presented both. The incidence of transients approaches 100% as the duration of the imaging period increases, providing a basis for the

high penetrance of their effects. Values are means $\pm$ s.e.m. for $n = 9$ –24 neurons (≥ 3 cultures).

METHODS. Neuron-enriched cultures were prepared from neural-plate-stage *Xenopus* embryos³⁰ as described¹⁸; cells were grown in tissue culture dishes in fully defined culture medium containing 116 mM NaCl, 0.67 mM KCl, 1.31 mM MgSO_4 , 10 mM CaCl_2 and 4.6 mM Tris; pH was adjusted to 7.8 with HCl. To examine fluctuations in Ca_i^{2+} , neurons were loaded with 2 μM Fluo-3-AM (Molecular Probes) and imaged at 10-s intervals for extended periods with a Bio-Rad MRC600 argon laser confocal system with a Zeiss microscope and $\times 20$ water-immersion objective. Images were recorded during two sequential 5-h intervals, from 7–12 h and 12–17 h *in vitro*. The low rate of image capture coupled with continuous superfusion of culture medium at $\sim 1 \text{ ml min}^{-1}$ facilitated examination of Ca^{2+} transients for these long periods. Additional imaging during the period from 3–7 h and for 1-h periods between 19–21 h revealed overall patterns of activity of spontaneous calcium spikes and waves occurring in somata and growth cones. Neurons continued to differentiate after imaging. Fluorescent pixel intensities were analysed with the IMAGE program (W. Rasband, NIH); changes were normalized to baseline, and spikes and waves were scored as events $>150\%$ of baseline fluorescence (F/F_0) and distinguished on the basis of their kinetics and pharmacology¹⁸.

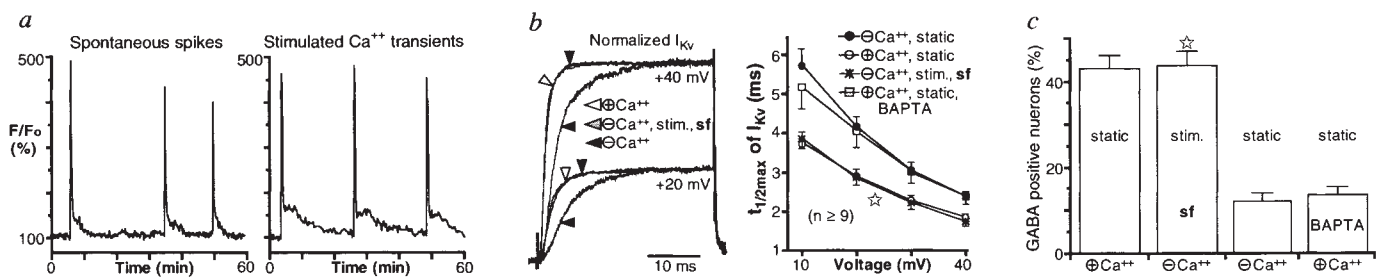
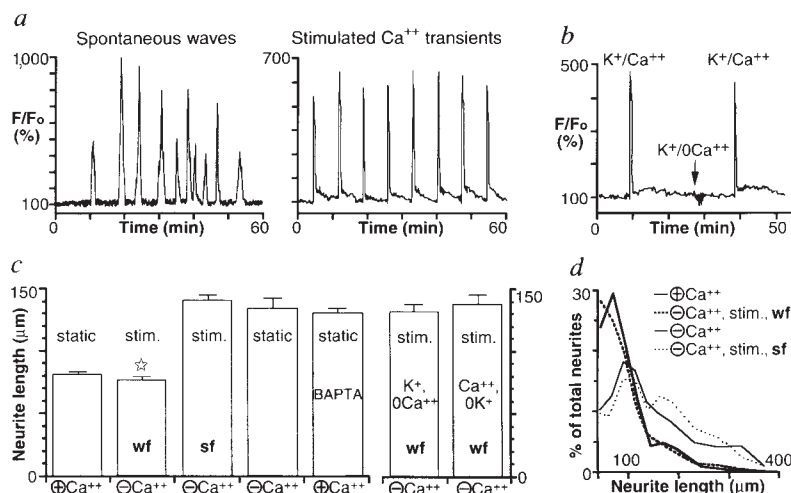


FIG. 2 Stimulation of calcium elevations in spontaneous spike patterns promotes potassium current maturation and neurotransmitter accumulation. *a*, In a neuronal soma, Ca_i^{2+} elevations are generated by application of 20–30 s pulses of high KCl with Ca^{2+} at 3 per h, which mimic spontaneous Ca^{2+} spikes. F/F_0 indicates fluorescence increase above baseline. Eight hours in culture. Fura-2 measurements of peak Ca_i^{2+} elevation in somata during spikes¹⁸ are consistent with values achieved by high K^+ - Ca^{2+} stimulation (530 ± 50 versus 650 ± 40 nM); spike durations are briefer than those of stimulated transients (9 ± 1 versus 20 ± 1 s; $n \geq 9$). *b*, Standard maturation of kinetics of activation of I_{Kv} is achieved by elevations of Ca_i^{2+} at natural spike frequencies (sf, 3 per h for 5 h, starting at 5 h in culture, and 1 per h for the next 7 h). Normalized records of I_{Kv} from three neurons, grown $\pm$ extracellular Ca^{2+} and in a stimulated culture (left; arrows). $t_{1/2\text{max}}$ at each voltage is prolonged by growth in the absence of Ca^{2+} or by BAPTA in the presence of Ca^{2+} ; it is restored by stimulated Ca^{2+} transients (*; right). *c*, Normal expression of GABA is also achieved by periodic elevation of Ca_i^{2+} at spike frequencies (sf, *); BAPTA suppresses development of GABA immunoreactivity as effectively as growth in the absence of Ca^{2+} , indicating that elevation of Ca_i^{2+} is required.

Means $\pm$ s.e.m. for $n \geq 4$ cultures, ≥ 50 neurons per dish.

METHODS. Cells were cultured in 0.25 ml Ca^{2+} -free medium using a volume reducer and continuously superfused with saline at 2.5 ml min^{-1} . The composition of salines was automatically switched for brief periods by computer controlled solenoid valves (General Valve Corp.). Cells were loaded with Fura-2 by the same procedure as with Fluo-3 and calibrated as described¹⁸. I_{Kv} was elicited as before¹³ using an Axopatch amplifier (Axon Instruments) to hold neurons at -40 mV and depolarize them to voltages ranging from $+10$ to $+40 \text{ mV}$ for 30 ms; all recordings were made in the same saline, containing 10 mM Co^{2+} to block I_{Ca} . Experimental and control values were obtained from cultures from the same neural plate¹⁹. GABA immunoreactivity was assessed on buffered 0.3% glutaraldehyde–4% paraformaldehyde-fixed cultures using a rabbit primary polyclonal antibody and a biotinylated goat anti-rabbit secondary antibody followed by avidin-horseradish peroxidase and diaminobenzidine²¹. Neurons were loaded with 2 μM BAPTA-AM for 1.5 h during dissociation of the neural plate before plating. Effects of BAPTA are unlikely to be due to generation of toxic metabolites, because longer exposure to the AM ester of Fluo-3 does not produce similar effects.

FIG. 3 Calcium elevations driven in spontaneous wave patterns regulate neurite length. **a**, In a growth cone, Ca^{2+} transients generated by stimuli delivered at 8 per h mimic spontaneous Ca^{2+} waves. These transients increase F/F_0 to $550 \pm 15\%$, with a duration of 27 ± 2 s, whereas spontaneous waves in growth cones increase F/F_0 to $520 \pm 30\%$, with a duration of 33 ± 4 s ($n \geq 50$). **b**, Stimulation with high KCl alone without CaCl_2 does not elevate Ca^{2+} . **a**, **b**, 8 h in culture. Similar results were obtained by stimulation with CaCl_2 alone (data not shown). **c**, Ca^{2+} transients elicited at wave frequencies (wf, 8 per h for 12 h starting at 6 h in culture) are sufficient to regulate neurite extension ($\star$), whereas spike frequencies (sf) are ineffective, although normal maturation of I_{Kv} was observed in these neurons. BAPTA enhances neurite outgrowth to the same extent as growth in the absence of Ca^{2+} , indicating regulation by Ca^{2+} transients (left). Neurites are long following stimulation with pulses of KCl or CaCl_2 alone at wave frequencies (right). Mean $\pm$ s.e.m. for $n \geq 100$ neurons (≥ 3 cultures). **d**, The distributions of neurite lengths are similar between cultures in Ca^{2+} -containing medium and Ca^{2+} -free medium stimulated at wave frequency, and between Ca^{2+} -free medium and Ca^{2+} -free medium stimulated at spike frequencies, respectively. Bin widths on the abscissa correspond to inflection points on the curves. Analysis of extension of



individual processes indicates that axon outgrowth is inhibited in 60% of cells by stimulation at wave frequencies (unpublished data).

Interestingly, stimulation at spike frequencies is not sufficient to regulate neurite extension, and neurite length is similar to that in Ca^{2+} -free cultures ($P=0.46$). However, stimulation mimicking the wave frequency of growth cones (Fig. 1b, 3a) restricts extension of neurites to lengths observed in Ca^{2+} -containing cultures at 1 day ($P=0.78$; Fig. 3c). The shifted distribution of neurite lengths in response to wave stimulation matches the distribution seen in Ca^{2+} -containing medium (Fig. 3d). Depolarizing stimuli have been shown to affect neuronal differentiation, including neurite extension^{12,19,20}. To evaluate the effects of depolarization and external Ca^{2+} separately, we perfused pulses of high KCl without Ca^{2+} (Fig. 3b) or CaCl_2 without high KCl. Neurite lengths in either case are the same as those achieved by growth in Ca^{2+} -free cultures (Fig. 3c), implying that depolarization exerts its effects by elevation of Ca^{2+} .

The effects of stimulated Ca^{2+} transients in Ca^{2+} -free medium suggest that spikes and waves are differentially sufficient to direct separate aspects of neuronal development. We thus expected suppression of spontaneous transients to make differentiation of

neurons in Ca^{2+} -containing medium similar to that in Ca^{2+} -free medium. This hypothesis was tested by growing cells in Ca^{2+} -containing medium after loading them with BAPTA, a rapid Ca^{2+} chelator that does not affect baseline Ca^{2+} levels in somata (~ 50 nM) or growth cones (~ 35 nM) over 16 h, assessed with Fura-2AM. However, the chelator suppresses elevation of Ca^{2+} with KCl stimuli during this period, both in somata (from 650 to 150 nM) and in growth cones (from 270 to 100 nM). BAPTA prevents maturation of I_{Kv} and appearance of GABA (Fig. 2b, c), but accelerates outgrowth of neurites (Fig. 3c). These results indicate that intracellular Ca^{2+} transients are necessary for differentiation and imply the existence of threshold amplitudes for the effects of spikes and waves at specific frequencies.

Developmental regulation of spikes and the prominence of waves in growth cones suggests that frequency is a physiologically relevant parameter encoding differentiation. To test this, Ca^{2+} -free cultures were stimulated at a range of frequencies mimicking the patterns of those naturally occurring for spikes and

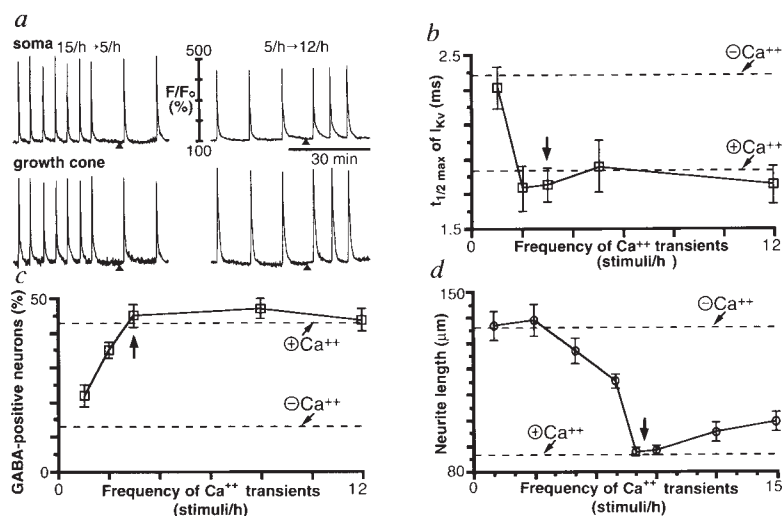


FIG. 4 Turning curves reveal stimulation of differentiation by natural frequencies of Ca^{2+} transients. Dashed lines indicate values attained in static culture with or without Ca^{2+} . **a**, Amplitudes and durations of stimulated transients appear constant over a range of stimulation frequencies. Records from soma and growth cone of 2 neurons at 12 h in culture, stimulated at 15 per h and then 5 per h (left), and at 5 per h then 12 per h (right); $\blacktriangle$ indicates the shift in stimulation frequency. Responses to stimuli at one or two frequencies in single cells vary randomly by $<10\%$ in amplitude, from 12–35 s in duration and by $<25\%$ in time integral ($\Delta F/F_0 \cdot \text{min}$; $3\text{--}15 \text{ h}^{-1}$, $n \geq 15$). Mean time integrals for naturally occurring somatic spikes (270 ± 25) and waves in growth cones (413 ± 32 ; $n \geq 50$) are comparable to stimulated transients at these locations (285 ± 21 , 385 ± 19 ; $n = 70$). **b**, Normal maturation of I_{Kv} is stimulated by Ca^{2+} transients at a frequency of 2 per h or greater, consistent with mean spike frequencies. **c**, Normal appearance of GABA shows a similar frequency dependence. **d**, Regulation of neurite outgrowth is optimized at spontaneous frequencies of waves arising in growth cones. **b–d**, Means $\pm$ s.e.m. for $n \geq 11$ neurons (**b**); $n \geq 5$ cultures (**c**); $n \geq 150$ neurons (**d**). Transients appear to affect channel modulation and neurite extension in all neurons, but to stimulate expression of GABA in only a subset of cells.

for waves. Peaks, durations and time integrals of stimulated Ca^{2+} transients are relatively constant at different frequencies (Fig. 4a). For spikes, stimulation at 2 per h or higher for the first 5 h and at a lower frequency (one third the initial value) for the next 7 h is effective in promoting maturation of I_{Kv} (Fig. 4b). Similar stimulation shows that appearance of GABA is also keyed to these frequencies, which lie at or above mean values for spikes (Fig. 4c). Neurite extension appears more tightly linked to frequencies of Ca^{2+} transients naturally occurring in growth cones. These data explain the absence of effect of spike suppression on neurite length¹⁸, because their addition to the spontaneous frequency of waves in growth cones would have no significant impact on neurite extension (Fig. 4c). Stimulation at steady frequencies of 8–9 per h optimally regulates neurite length, whereas frequencies of either 1–7 per h or 15 per h are significantly less effective (Fig. 4d; $P < 0.02$). A mechanism that counts the number of transients does not account for frequency-dependent differentiation. Delivery of 15 transients between 6–7 or 9–10 h in Ca^{2+} -free medium (equivalent to a spike pattern with 2 per h for 5 h and 0.7 per h for 7 h) fails to promote maturation of I_{Kv} ; reversal of the 12 h distributed pattern is also ineffective ($t_{1/2\text{max}}$ values at +40 mV are 2.6 ± 0.2 , 2.7 ± 0.4 , and 2.5 ± 0.2 ms, significantly different from 1.8 ± 0.1 ms in Ca^{2+} -containing medium, mean $\pm$ s.e.m. for $n \geq 7$, $P < 0.01$). Suppression of protein kinase C with 250 nM phorbol myristyl acetate (PMA) blocks maturation of I_{Kv} in Ca^{2+} -containing medium, whereas stimulation of protein kinase C with 10 nM PMA prevents suppression of I_{Kv} in Ca^{2+} -free medium¹³. The same treatments have no effect on neurite length (86 ± 2 ; 82 ± 2 μm ; 135 ± 3 ; 130 ± 5 μm), suggesting that spikes and waves decode information by different mechanisms.

Our results imply that spontaneous Ca^{2+} transients are both sufficient and necessary to drive normal differentiation, and that separate aspects are encoded in distinct frequencies of spikes and waves^{21,22}. Alteration in transient frequency alone, without substantial variation in amplitude, duration and time integral, is sufficient to affect expression of specific phenotypes. Moreover, the frequencies effective in achieving normal differentiation are those occurring naturally. Stimulation studies provide significant insights into the functions of transients^{9,23}. The importance of mimicking natural patterns of activity in developing neurons suggests that they are programmed to exhibit particular frequency patterns of Ca^{2+} transients, and raises intriguing issues concerning the mechanisms by which isolated neurons regulate the frequency of these events. Our findings may be pertinent to Ca^{2+} transients naturally generated by other developing neurons, different cell types and by glia^{24–29}. As in other cells, Ca^{2+} transients in *Xenopus* neurons appear to activate protein kinases¹³ and may stimulate transcription. Observation of spontaneous spikes and waves in the intact spinal cord¹⁸ makes it likely that they exert similar effects *in vivo*. Further studies will be necessary to identify the molecular triggers that elicit both classes of Ca^{2+} transients at functionally effective frequencies and the biochemical cascades by which they cue particular aspects of neuronal development. □

15. Bixby, J. L. & Spitzer, N. C. *Dev. Biol.* **106**, 89–96 (1984).
16. Holliday, J. & Spitzer, N. C. *Dev. Biol.* **141**, 13–23 (1990).
17. Holliday, J., Adams, R. J., Sejnowski, T. J. & Spitzer, N. C. *Neuron* **7**, 787–796 (1991).
18. Gu, X., Olson, E. C. & Spitzer, N. C. *J. Neurosci.* **14**, 6325–6335 (1994).
19. Shatz, C. J. *Neuron* **5**, 745–756 (1990).
20. Kocsis, J. D., Rand, M. N., Lankford, K. L. & Waxman, S. G. *J. Neurobiol.* **25**, 252–264 (1994).
21. Rink, T. J. & Jacob, R. *Trends Neurosci.* **12**, 43–46 (1989).
22. Meyer, T. & Stryer, L. A. *Rev. Biophys. biophys. Chem.* **20**, 153–174 (1991).
23. Turrigiano, G., Abbott, L. F. & Marder, E. *Science* **264**, 974–977 (1994).
24. Yuste, R., Peinado, A. & Katz, L. C. *Science* **257**, 665–669 (1992).
25. Komuro, H. & Rakic, P. *Science* **257**, 806–809 (1992).
26. Komuro, H. & Rakic, P. *Science* **260**, 95–97 (1993).
27. Poenie, M., Alderton, J., Tsien, R. Y. & Steinhardt, R. A. *Nature* **315**, 147–149 (1985).
28. Flucher, B. E. & Andrews, S. B. *Cell Motil. Cytoskel.* **25**, 143–157 (1993).
29. Fatatis, A. & Russell, J. T. *Glia* **5**, 95–104 (1992).
30. Nieuwkoop, P. D. & Faber, J. *Normal Table of Xenopus laevis* 2nd edn (North Holland, Amsterdam, 1967).

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Expression of a Delta homologue in prospective neurons in the chick

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THE product of the *Delta* gene, acting as ligand, and that of the *Notch* gene, acting as receptor, are key components in a lateral-inhibition signalling pathway that regulates the detailed patterning of many different tissues in *Drosophila*^{1–8}. During neurogenesis in particular, neural precursors, by expressing *Delta*, inhibit neighbouring *Notch*-expressing cells from becoming committed to a neural fate^{5,9,10}. Vertebrates are known to have several *Notch* genes^{11–14}, but their functions are unclear and their ligands hitherto unidentified. Here we identify and describe a chick *Delta* homologue, *C-Delta-1*. We show that *C-Delta-1* is expressed in prospective neurons during neurogenesis, as new cells are being born and their fates decided. Our data from the chick, combined with parallel evidence from *Xenopus*¹⁵, suggest that both the *Delta*/*Notch* signalling mechanism and its role in neurogenesis have been conserved in vertebrates.

We identified a chick *Delta* homologue, *C-Delta-1*, using the polymerase chain reaction (PCR) and degenerate oligonucleotide primers (Fig. 1). The DNA sequence of *C-Delta-1* corresponds to a protein of 722 amino acids, structurally homologous to *Drosophila* *Delta* (Fig. 1a, b) and distinct from vertebrate homologues of the *Delta*-related Serrate protein (our unpublished results). *C-Delta-1* contains a putative transmembrane domain, a signal sequence and eight epidermal-growth-factor (EGF)-like repeats in its extracellular region (one repeat less than *Drosophila* *Delta*). The amino-terminal domain of *C-Delta-1* is closely related to a similar domain in the fly *Delta* protein, which is described as necessary and sufficient for *in vitro* binding to *Notch*⁸. This conserved region includes the so-called DSL motif (Fig. 1b)¹⁶, shared by all known members of the family of presumed ligands of *Notch*-like proteins (*Delta* and *Serrate* in *Drosophila*; *Lag-2* and *Apx-1* in *Caenorhabditis elegans*)^{16–20}. A second cysteine-rich N-terminal region is conserved between the fly and chick proteins, but absent from the related *C. elegans* proteins (Fig. 1b). We have also cloned the

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1. Colbran, R. J. & Soderling, T. R. *Curr. Top. Cell Regulation* **31**, 181–221 (1990).
2. Braha, O., Edmonds, B., Sacktor, T., Kandel, E. R. & Klein, M. J. *Neurosci.* **13**, 1839–1851 (1993).
3. Ghosh, A., Carnahan, J. & Greenberg, M. E. *Science* **263**, 1618–1623 (1994).
4. Sheng, M., McFadden, G. & Greenberg, M. E. *Neuron* **4**, 571–582 (1990).
5. Dash, P. K., Karl, K. A., Colicos, M. A., Prywes, R. & Kandel, E. R. *Proc. natn. Acad. Sci. U.S.A.* **88**, 5061–5065 (1991).
6. Lerea, L. S. & McNamara, J. O. *Neuron* **10**, 31–41 (1993).
7. Bading, H., Ginty, D. D. & Greenberg, M. E. *Science* **260**, 181–186 (1993).
8. Ensten, H. et al. *J. Biol. Chem.* **269**, 15520–15527 (1994).
9. Sheng, H. Z., Fields, R. D. & Nelson, P. G. *J. Neurosci. Res.* **35**, 459–467 (1993).
10. Cohan, C. S. & Kater, S. B. *Science* **232**, 1638–1640 (1986).
11. Cline, H. T. & Tsien, R. W. *Neuron* **6**, 259–267 (1991).
12. Fields, R. D., Neale, E. A. & Nelson, P. G. *J. Neurosci.* **10**, 2950–2964 (1990).
13. Desarmenien, M. G. & Spitzer, N. C. *Neuron* **7**, 797–805 (1991).
14. Spitzer, N. C., Debaca, R. C., Allen, K. A. & Holliday, J. J. *comp. Neurol.* **337**, 168–175 (1993).

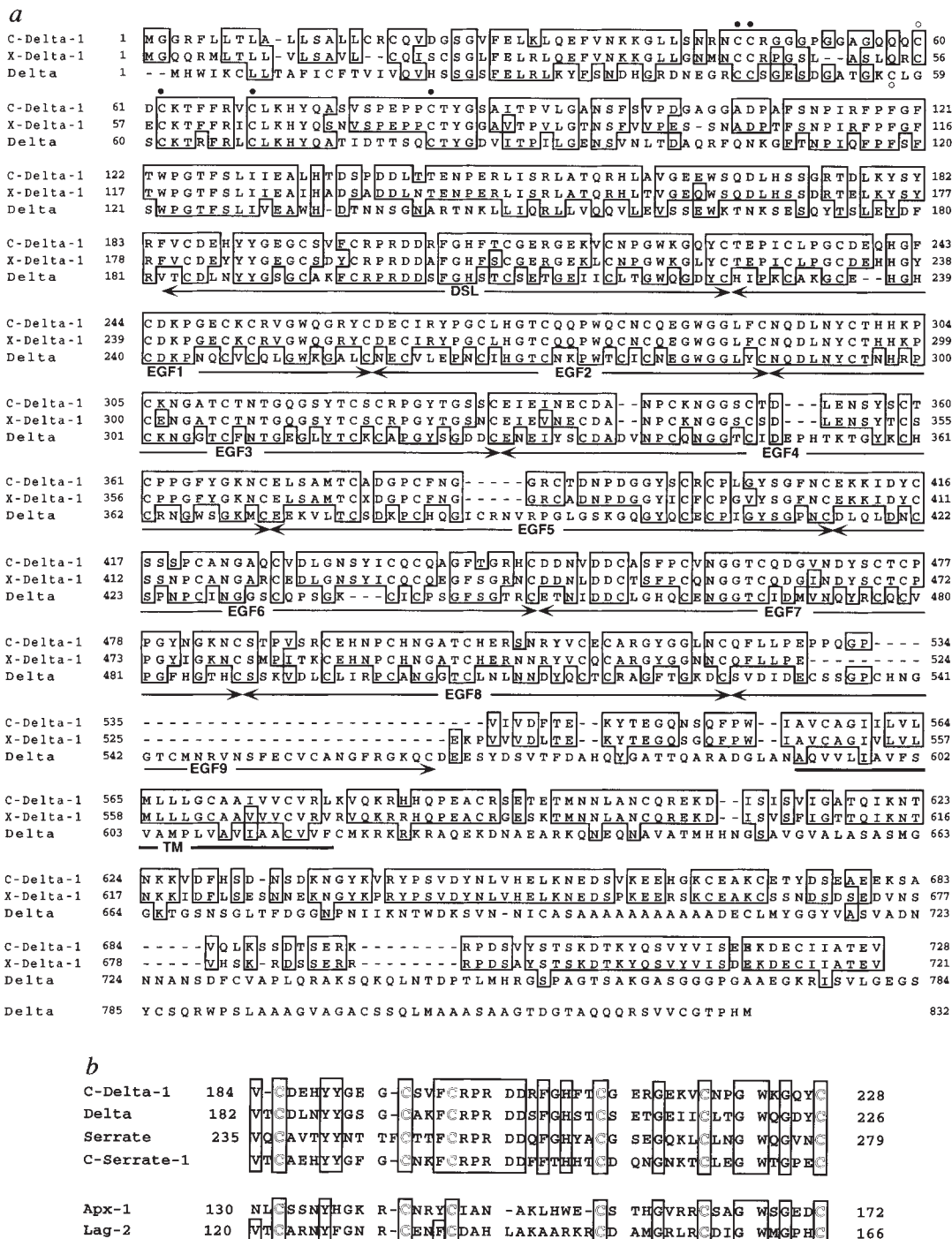
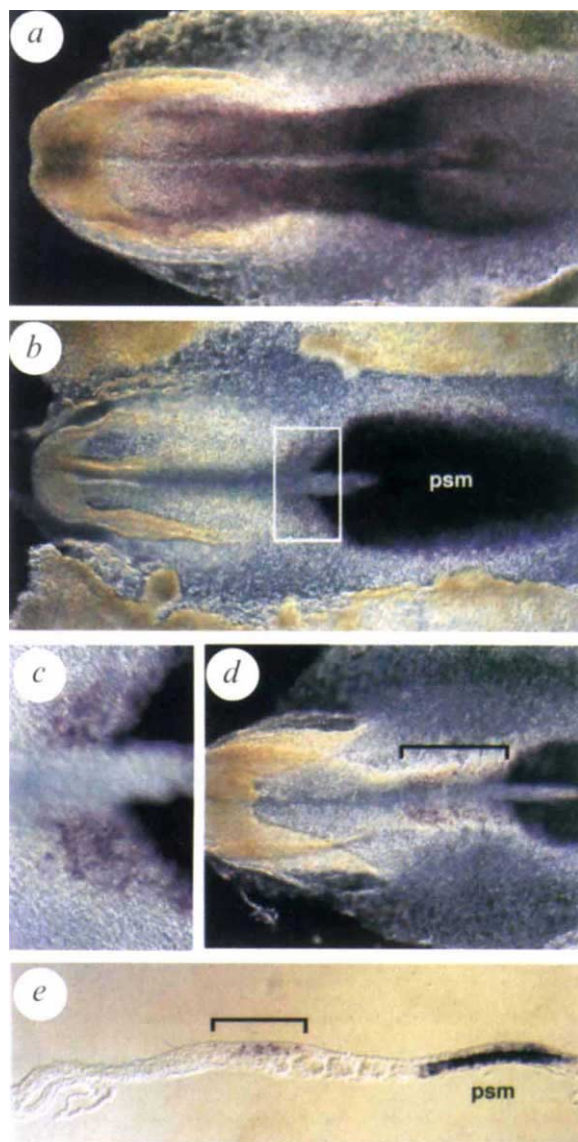


FIG. 1 a, Predicted protein sequence of C-Delta-1, aligned with that of *Drosophila* Delta and X-Delta-1 (ref. 15), indicating the conserved domain structures: EGF repeats, DSL domain, and transmembrane domain (TM). Conserved amino acids are boxed, filled and open circles denote aligned and non-aligned N-terminal cysteine residues, respectively. Although the intracellular domains of C-Delta-1 and X-Delta-1 closely resemble each other, they show no significant homology to the corresponding part of *Drosophila* Delta. b, Alignment of DSL domains from C-Delta-1, Delta^{1,2}, Serrate^{17,18}, C-Serrate-1 (A.M., D.H., D.L.H. and J.L., manuscript in preparation), Apx-1 (ref. 20) and Lag-2 (ref. 16, 19), showing the conservation of cysteine spacings, of amino acids that are conserved between presumed ligands for Notch-like proteins in *Drosophila* and vertebrates, and of those further conserved in *C. elegans* ligands (boxes).

METHODS. C-Delta-1 was cloned by PCR using the degenerate oligonucleotide primers TTCGGITT(C/T)ACITGGCCIGGIAC and TCIATGCAIG-TICCCIC(A/G)TT which correspond to the fly Delta protein sequences FGFTWPGT and NGGTCID, respectively^{1,2}. The initial reaction used

50 ng of first-strand oligo d(T)-primed cDNA from stage 4-6 embryos, 1 µg of each primer, 0.2 mM dNTPs, 2U of Taq polymerase, in 50 µl of the supplied buffer (Perkin-Elmer). 40 cycles of amplification were performed at 94 °C for 30 s; 50 °C for 2 min; 72 °C for 2 min. Amplified DNA fragments were separated on an agarose gel, cloned in Bluescript pKS- (Stratagene) and sequenced. Two Delta homologues were identified, one of which (C-Delta-1) is expressed in the nervous system. DNA fragments corresponding to C-Delta-1 were used to screen a stage-17 spinal cord cDNA library and several full-length clones were obtained and sequenced. We amplified DNA fragments from the chick C-Notch-1 gene by similar methods (data not shown); partial sequence data and pattern of expression indicate close similarity to the rodent Notch-1 gene¹²⁻¹⁴. An initial X-Delta-1 PCR fragment was isolated from stage-17 (neurula) embryos by similar methods, and used to screen a stage-17 embryonic cDNA library²⁵, yielding a 3.2-kb cDNA (pXDL-1) which contains the entire protein-coding region. Sequences were analysed using the Wisconsin GCG set of programs.



Xenopus Delta-1 homologue, *X-Delta-1* (ref. 15), which encodes a protein that is 81% identical to *C-Delta-1* and has all the structural motifs described above (Fig. 1).

The structural conservation between the chick and fly *Delta* proteins, including domains identified as critical for Notch binding⁸, suggests that *C-Delta-1* functions as a ligand for a chick Notch protein, and that a *Delta*/Notch-mediated mechanism of lateral inhibition might operate in the chick. During *Drosophila* neurogenesis, *Delta* is transiently expressed in neural precursors, inhibiting neighbouring *Notch*-expressing cells from also becoming neural^{21,22}. If *C-Delta-1* acts similarly during chick neurogenesis, it should also be transiently expressed in neuronal precursor cells, while these are becoming determined. An analysis of *C-Delta-1* expression in the developing CNS indicates that this is indeed the case.

<i>f</i>	Hamburger-Hamilton Stage (nominal age in h; somite nos.)		
	End final S-phase	Initial <i>C-Delta-1</i> expression	Initial NF expression
Mid/posterior Hindbrain	4 (19h; 0)	6 (24h; 0)	9 (31h; 7-9)
Spinal cord, somites 5-8	6 (24h; 0)	8 (28h; 4-6)	10 (36h; 10-12)
Forebrain/ Midbrain	7 (25h; 1-3)	8 (28h; 4-6)	10 (36h; 10-12)
Spinal cord, somites 9-12	8 (28h; 4-6)	9 (31h; 7-9)	11 (43h; 13-15)

FIG. 2 *C-Delta-1* and *C-Notch-1* expression correlate with onset of neurogenesis in the one-day (E1) neural plate. Anterior is to the left. Whole-mount *in situ* hybridization specimens are shown in a-d; e is a section. a, At stage 7, *C-Notch-1* is expressed throughout most of the neural plate and part of the underlying presomitic mesoderm. b, *C-Delta-1* at stage 7 is already detectable in the neural plate, in the future posterior hindbrain, just anterior to the first somite (white box). The posterior end of this neural domain is roughly level with the anterior margin of a domain of very strong expression in the underlying presomitic mesoderm (psm). Earlier expression in the neural plate may occur and be masked by expression in the underlying mesoderm (our unpublished results). c, Higher-magnification view of the area boxed in b, showing scattered cells in the neural plate expressing *C-Delta-1*. d, At stage 8, *C-Delta-1* expression in the neural plate extends posteriorly as the neural plate develops. The domain of labelled neural plate cells visible in this photograph (bracketed) continues posteriorly over the presomitic mesoderm. e, Parasagittal section of a stage-8 embryo showing that *C-Delta-1* is expressed in scattered cells of the neural plate (dorsal layer of tissue; bracketed), and broadly in the presomitic mesoderm (ventral layer). The plane of section is slightly oblique, missing the posterior part of the neural plate domain (compare with d). f, Appearance of *C-Delta-1* expression closely follows the withdrawal of the first neuronal precursors from the division cycle and precedes the appearance of neurofilament (NF) antigen in the resultant differentiating neurons. Mid-hindbrain comprises rhombomeres 4-6, the level of the otic primordium; posterior hindbrain includes rhombomeres 7 and 8, and the levels of somites 1-4. Data for the timing of withdrawal from cell division and for neurofilament expression are taken from ref. 23. METHODS. Whole-mount *in situ* hybridization was performed by a modification of published methods (D.H. and D.I.H., in preparation). In summary, formaldehyde-fixed embryos were treated with protease and refixed with 4% formaldehyde/0.1% glutaraldehyde. Hybridization with DIG-labelled RNA probes was performed under stringent conditions (1.3 × SSC, 50% formamide at 65 °C, pH 5) in a buffer containing 0.2% Tween-20 and 0.5% CHAPS. Washed embryos were treated with Boehringer blocking reagent and incubated overnight in alkaline phosphatase-coupled anti-DIG antibody. After extensive washes, embryos were stained from 30 min to 16 h. Detailed protocols are available by anonymous ftp from ftp://lif.icnet.uk/icrf-public/recipes/insiturecipe11_95.hqx (Macintosh Word v5.1); or [insiturecipe11_95.rtf](ftp://lif.icnet.uk/icrf-public/recipes/insiturecipe11_95.rtf) (Microsoft Rich Text Format). The embryo in e was wax-sectioned after hybridization.

C-Delta-1 expression in the neural plate is first detected at stage 6-7 (31 h, 0/1 somite) in scattered cells just anterior to the presomitic mesoderm (Fig. 2b, c). This region gives rise to the mid/posterior hindbrain, where the earliest differentiated CNS neurons are first detected by a neurofilament antibody at stage 9 (31 h, 7-9 somites)²³, 6 h after the initial *C-Delta-1* expression (Fig. 2f). As neurogenesis proceeds, expression of *C-Delta-1* continues to foreshadow the spatio-temporal pattern of neuronal differentiation (Fig. 2f), spreading posteriorly along the spinal cord and anteriorly into the midbrain and forebrain (Fig. 2d, e). For example, the most posterior expressing cells in the stage 8 spinal cord are at the level of the prospective sixth somite, 6-8 h before the first neurons at that level express neurofilament antigen²³ (Fig. 2f). In all cases, *C-Delta-1* is expressed

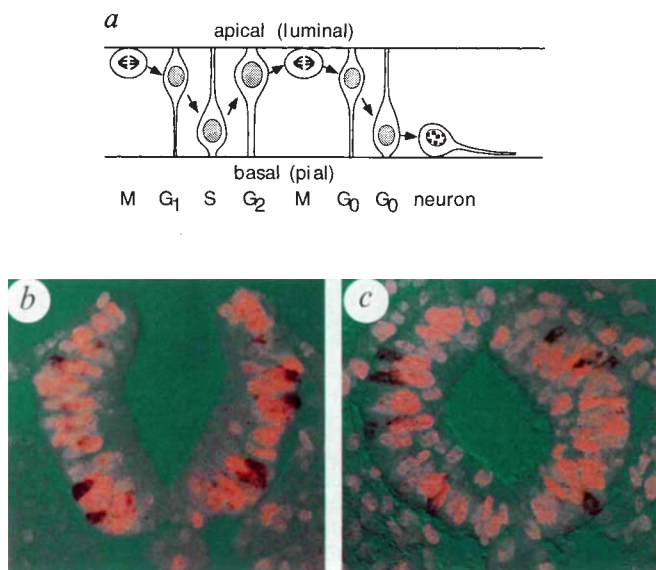


FIG. 3 *C-Delta-1*-expressing cells do not incorporate BrdU. Of 612 *C-Delta-1*⁺ cells, 581 were BrdU⁻ (76 sections; 6 embryos). *a*, Diagram showing how phase in the cell cycle is related to apico-basal position of the nucleus for cells in the neuroepithelium; S-phase nuclei lie basally^{24,26}. Nuclei are indicated by shading. *b*, Section through the neural tube of a stage 9 embryo labelled for 2 h with BrdU showing *C-Delta-1* expressing cells (dark brown on blue background) and BrdU-labelled nuclei (pink). Labelled nuclei are predominantly basal, where DNA synthesis occurs, yet basal *C-Delta-1*-expressing cells are unlabelled. *c*, Section through a stage-9 embryo incubated for 4 h: many labelled nuclei have exited S-phase and have moved towards the lumen, but *C-Delta-1*-expressing cells are still basal and not labelled with BrdU. METHODS. 75 μ l 0.1 mM BrdU in PBS was dropped onto stage 7–9 embryos which were incubated at 38 °C for 2–4 h before fixation for *in situ* hybridization. 15- μ m cryostat sections were hybridized with DIG-labelled RNA probes essentially according to ref. 27. After staining, slides were washed in PBS, and processed for BrdU immunodetection²⁸. Anti-BrdU (1:1,000; Sigma) was detected using fluorescein isothiocyanate-coupled goat anti-mouse secondary antibody (Cappel). *C-Delta-1* expression was examined by DIC microscopy and BrdU labelling by conventional and confocal fluorescence microscopy.

in scattered cells within domains of uniform *C-Notch-1* expression (Fig. 2*a*).

The localization and time course of *C-Delta-1* expression suggest that the gene is switched on at an early step in neurogenesis, and that the cells expressing *C-Delta-1* are prospective neurons that have not yet begun to display differentiation markers. To test this idea, we made use of observations²³ that prospective neurons are the only non-cycling cells in the early neural tube. They finish their final S phase 11–15 h before expressing neurofilament antigen (Fig. 2*f*) and their nuclei, after completing a last mitosis, adopt a characteristic location near the basal surface of the neuroepithelium, where all the other cell nuclei are in S-phase^{23,24} (Fig. 3*a*).

We labelled stage 7–9 embryos with bromodeoxyuridine (BrdU), and double-stained for BrdU incorporation and *C-Delta-1* expression. 95% of the *C-Delta-1*-expressing cells were unlabelled, with their nuclei predominantly located near the basal surface, where most other nuclei were BrdU-labelled (Fig. 3*b*, *c*). These results imply that *C-Delta-1* is expressed in cells that have withdrawn from the cell cycle and must indeed be prospective neurons. The few BrdU⁺/*C-Delta-1*⁺ cells have their nuclei outside the basal zone; these may be cells that finished their final S-phase soon after exposure to BrdU, moved apically

to complete their final mitosis, and switched on *C-Delta-1* expression.

C-Delta-1 is also expressed in the later neural tube and peripheral nervous system. Again, the timing of expression and the location of the expressing cells imply that they are neuronal precursors that have not yet begun to differentiate (unpublished observations). Thus, *C-Delta-1* expression appears to be the earliest known marker for prospective neurons.

The accompanying paper¹⁵ demonstrates the function of the *Xenopus* homologue of *C-Delta-1* during primary neurogenesis: *X-Delta-1* expression in a subset of the neuroepithelial cells inhibits their neighbours from adopting a neuronal fate. Preliminary evidence indicates that *C-Delta-1* has a similar inhibitory action when expressed in *Xenopus* embryos (unpublished observations). We propose that *C-Delta-1*, like its *Drosophila* and *Xenopus* counterparts, mediates lateral inhibition throughout neurogenesis to restrict the proportion of cells that, at any time, become committed to a neural fate. Delta/Notch signalling may thus be a universal mechanism for regulating nerve cell production.

Note added in proof: Since this paper was submitted, it has been reported²⁹ that *Jagged*, a *Serrate*-related gene that appears to be the rat homologue of the chick *C-Serrate-1* gene (Fig. 1*b*), encodes a product that can interact with vertebrate Notch receptors. □

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- Vässen, H., Bremer, K. A., Knust, E. & Campos-Ortega, J. A. *EMBO J.* **6**, 3431–3440 (1987).
- Kopczynski, C. C., Alton, A. K., Fechtel, K., Kooh, P. J. & Muskavitch, M. A. *Genes Dev.* **2**, 1723–1735 (1988).
- Fehon, R. G. et al. *Cell* **61**, 523–534 (1990).
- Artavanis-Tsakonas, S. & Simpson, P. *Trends genet. Sci.* **7**, 403–407 (1991).
- Heitzler, P. & Simpson, P. *Cell* **64**, 1083–1092 (1991).
- Greenwald, I. & Rubin, G. M. *Cell* **68**, 271–281 (1992).
- Fortini, M. E. & Artavanis-Tsakonas, S. *Cell* **75**, 1245–1247 (1993).
- Muskavitch, M. *Dev. Biol.* **166**, 415–430 (1994).
- Ghyssen, A., Dambly-Chaudière, C., Jan, L. Y. & Jan, Y. N. *Genes Dev.* **7**, 723–733 (1993).
- Campos-Ortega, J. in *The Development of Drosophila melanogaster* (eds Bate, M. & Martinez Arias, A.) 1091–1129 (Cold Spring Harbor Laboratory Press, New York, 1993).
- Coffman, C., Harris, W. & Kintner, C. *Science* **249**, 1438–1441 (1990).
- Weinmaster, G., Roberts, V. J. & Lemke, G. *Development* **113**, 199–205 (1991).
- Weinmaster, G., Roberts, V. J. & Lemke, G. *Development* **116**, 931–941 (1992).
- Lardelli, M. & Lendahl, U. *Exp. Cell Res.* **204**, 364–372 (1993).
- Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D. & Kintner, C. *Nature* **375**, 761–766 (1995).
- Tab, F. E., Yeagers, J. J. & Thomas, J. H. *Nature* **368**, 150–154 (1994).

- Fleming, R. J., Scottgale, T. N., Diederich, R. J. & Artavanis-Tsakonas, S. *Genes Dev.* **4**, 2188–2201 (1990).
- Thomas, U., Speicher, S. A. & Knust, E. *Development* **111**, 749–761 (1991).
- Henderson, S. T., Gao, D., Lambie, E. J. & Kimble, J. *Development* **120**, 2913–2924 (1994).
- Mello, C. C., Draper, B. W. & Priess, J. R. *Cell* **77**, 95–106 (1994).
- Haenlin, M., Kramatschek, B. & Campos-Ortega, J. A. *Development* **110**, 905–914 (1990).
- Kooh, P. J., Fehon, R. G. & Muskavitch, M. A. *Development* **117**, 493–507 (1993).
- Sechrist, J. & Bronner-Fraser, M. *Neuron* **7**, 947–963 (1991).
- Martin, A. H. & Langman, J. J. *Embryol. exp. Morphol.* **14**, 23–35 (1965).
- Kintner, C. R. & Melton, D. A. *Development* **99**, 311–325 (1987).
- Fujita, S. J. *comp. Neurol.* **120**, 37–42 (1963).
- Strähle, U., Blader, P., Adam, J. & Ingham, P. W. *Trends genet. Sci.* **10**, 75–76 (1994).
- Biffo, S., Verdun di Cantogno, L. & Fasolo, A. J. *Histochem. Cytochem.* **40**, 535–540 (1992).
- Lindsell, C. E., Shawber, C. J., Boulter, J. & Weinmaster, G. *Cell* **80**, 909–917 (1995).

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Absence of radius and ulna in mice lacking *hoxa-11* and *hoxd-11*

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MICE with targeted disruptions¹ in *Hox* genes have been generated to evaluate the role of the *Hox* complex in determining the mammalian body plan. This complex of 38 genes encodes transcription factors that specify regional information along the embryonic axes. Early in vertebrate evolution an ancestral complex shared with invertebrates was duplicated twice to give rise to the four linkage groups (*Hox* A, B, C and D)^{2,3}. As a consequence, corresponding genes on the separate linkage groups, called paralogues, are most closely related to each other. Based on sequence similarities, the *Hox* genes have been subdivided into 13 paralogous groups. The five most 5' groups (*Hox* 9–13) pattern the posterior region of the vertebrate embryo and the appendicular skeleton^{4–18}. Mice with individual mutations in the paralogous genes *hoxa-11* and *hoxd-11* have been described^{15–18}. By breeding these two strains together we have generated double mutants which have dramatic phenotypes not apparent in mice homozygous for the individual mutations. The radius and the ulna of the forelimb are almost entirely eliminated, the axial skeleton shows homeotic transformations, and there

are severe kidney defects not present in either single mutant. The limb and axial phenotypes are quantitative: as more mutant alleles are added to the genotype, the phenotype becomes progressively more severe. The appendicular skeleton defects suggest that paralogous *Hox* genes function together to specify limb outgrowth and patterning along the proximodistal axis.

For simplicity, we use 'A' (wild-type allele) and 'a' (mutant allele) to designate the *hoxa-11* genotype, and similarly 'D' and 'd' for *hoxd-11*. Compound heterozygotes (Aa; Dd) were crossed to each other to generate all nine genetic states. Four (aa; dd) adults were weaned from a total of 313 progeny. This is fewer than the 20 expected; most double mutants suffer perinatal death as a consequence of kidney dysfunction (see below). All other genotypes occurred in the expected Mendelian ratios.

The vertebrate forelimb is divided into three zones: the stylopod (humerus), zeugopod (radius and ulna), and autopod (carpals, metacarpals and phalanges) (Fig. 1a). The hindlimb is similarly arranged. All four limbs of the double mutants are severely affected. Synergistic effects of *hoxa-11* and *hoxd-11* are dramatic in the forelimb zeugopod wherein individual mutant homozygotes (aa; DD) and (AA; dd) show subtle malformations of the zeugopod (Fig. 1b, c), but, remarkably, the radius and ulna are almost entirely absent from double-mutant limbs (Fig. 1d). These extraordinary malformations result in a paw that is rotated 90° off its normal axis.

There are similar effects in the autopod. The wrist contains seven bones: three proximal carpals (pisiform, triangular and navicular lunate) and four distal carpals (d1–d4). In (aa; DD) and (AA; dd) forelimbs the proximal carpal bones are fused together^{15–17}. Although individual heterozygotes never show this defect, compound heterozygotes (Aa; Dd) do (Table 1). This suggests that any two mutant alleles (either both from *hoxa-11* or *hoxd-11*, or one from each locus) will cause carpal fusions. Genotypes (Aa; dd) and (aa; Dd), with three mutant alleles, have more severe defects of the proximal carpal bones in which the triangular and pisiform bones are grossly malformed, and the navicular lunate is slightly deformed (results not shown).

TABLE 1 Axial and carpal bone phenotypes of (*hoxa-11*; *hoxd-11*) mice

Genotype*	Axial column phenotypes			Carpal bone phenotypes			
	Phenotype†	Frequency (%)‡	Comments§	Genotype	Wild-type (%)	Carpal bone fusions¶	
(AA; DD)	T13:L6	100	wild-type	(AA; DD)	100		
(Aa; DD)	T13:L6	50	wild-type	(Aa; DD)	100		
	T12:L7	50	PHT				
(aa; DD)	T12:L8	50	PHT and AHT	(aa; DD)	33	17%	50%
	T12:L7:AL/s	50	PHT and AHT				
(AA; Dd)	T13:L6	100	wild-type	(AA; Dd)	100		
(AA; dd)	T13:L7	50	AHT	(AA; dd)		17%	17%
	T13:L6:AL/s	50	AHT				
(Aa; Dd)	T12:L7	33	PHT	(Aa; Dd)	17	17%	33%
	T13:L7	33	AHT				
	T12:AT/L:L6	17	PHT				
	T12:AT/L:L7	17	PHT and AHT				
(Aa; dd) and (aa; Dd)	T12:L8	67	PHT and AHT				
	T13:L7	33	AHT				
(aa; dd)	T12:L9	75	PHT and AHT				
	T12:L8:AL/s	25	PHT and AHT				

* A, *hoxa-11* wild-type allele; a, mutant allele; D, *hoxd-11* wild-type allele; d, mutant allele.

† T13:L6, wild-type pattern with 13 thoracics (T) followed by 6 lumbar (L). AL/s is an asymmetric vertebra: half is lumbar-like, half is sacral-like. AT/L is an asymmetric vertebra: half is thoracic-like, half is lumbar-like.

‡ Examined six animals for each genotype, except for (aa; dd) where only four were available.

§ PHT (posterior homeotic transformation); AHT (anterior homeotic transformation).

|| (Aa; dd) and (aa; Dd) data combined.

¶ Percentage of animals either showing a wild-type pattern of carpals (none fused) or a fusion between the navicular lunate (NL), triangular (T), and pisiform (P) carpal bones.

The distal carpals in these animals appear normal. With the addition of a fourth mutant allele, the autopod of the mouse is even more dramatically altered. All of the proximal carpal bones are affected: the pisiform is missing, the triangular is also either absent or is fused to the distal carpals, and the navicular lunare is deformed (Fig. 2a, b). The metacarpals are reduced in length, especially in digits II, III and V. In addition, digits I, II and III curve preaxially and are 'fused' together by the overlaying skin, perhaps owing to a failure of normally occurring cell death between the digits. The phalangeal bones P2 and P1 of digit III are often fused, and P2 is missing from digits II and V (Fig. 2a-d).

The zeugopodal phenotype is evident at E13.5 when the radius and ulna branch off from the humerus (Fig. 2e, f). At this stage the (aa; dd) zeugopod is already shorter than the wild type. In addition, the autopod condensations are altered. The wild-type autopod has initiated a full complement of digits, but condensa-

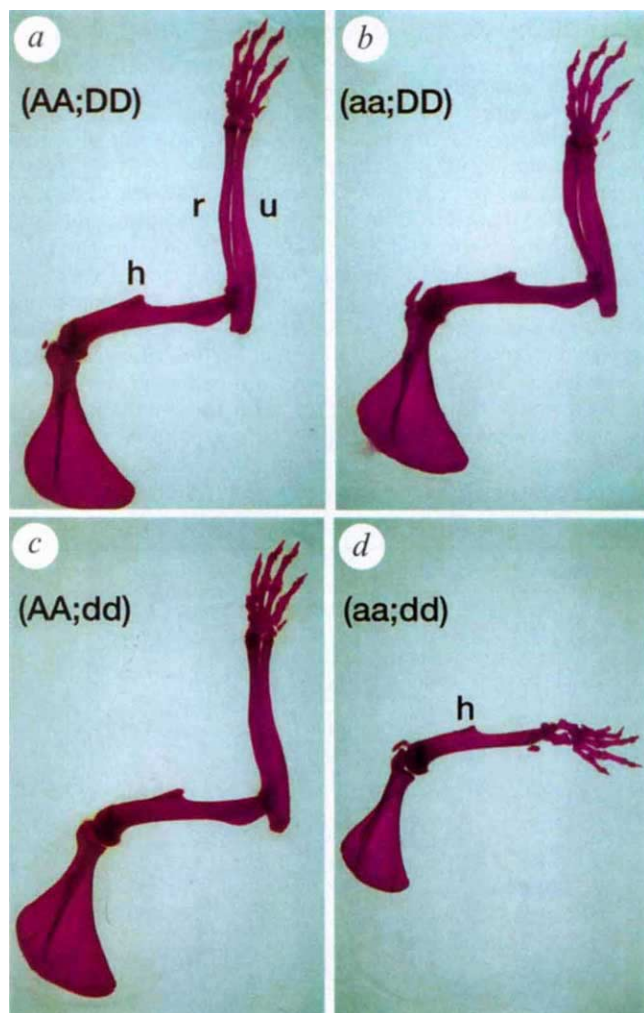


FIG. 1 Loss of the radius and ulna in the double-mutant mouse. Dorsal view of the right forelimbs from adult mouse skeleton preparations. a, Wild-type mouse (AA; DD) with a normal humerus (h), radius (r), and ulna (u). b, *Hoxa-11* mutant (aa; DD) with a slightly thicker and shorter zeugopod. c, *Hoxd-11* mutant (AA; dd) with subtle limb defects in the zeugopod and autopod¹⁶. d, The double mutant (aa; dd) showing an unexpected and dramatic reduction of the radius and ulna. The forepaw is also malformed and rotated 90° compared with wild type. Four (aa; dd) mice survived to adulthood and were examined. All four showed complete penetrance and expressivity of the limb phenotype. METHODS. All animals were genotyped by Southern blot analysis of tail DNA, and adult skeleton preparations were stained with Alizarin red as described^{15,16}.

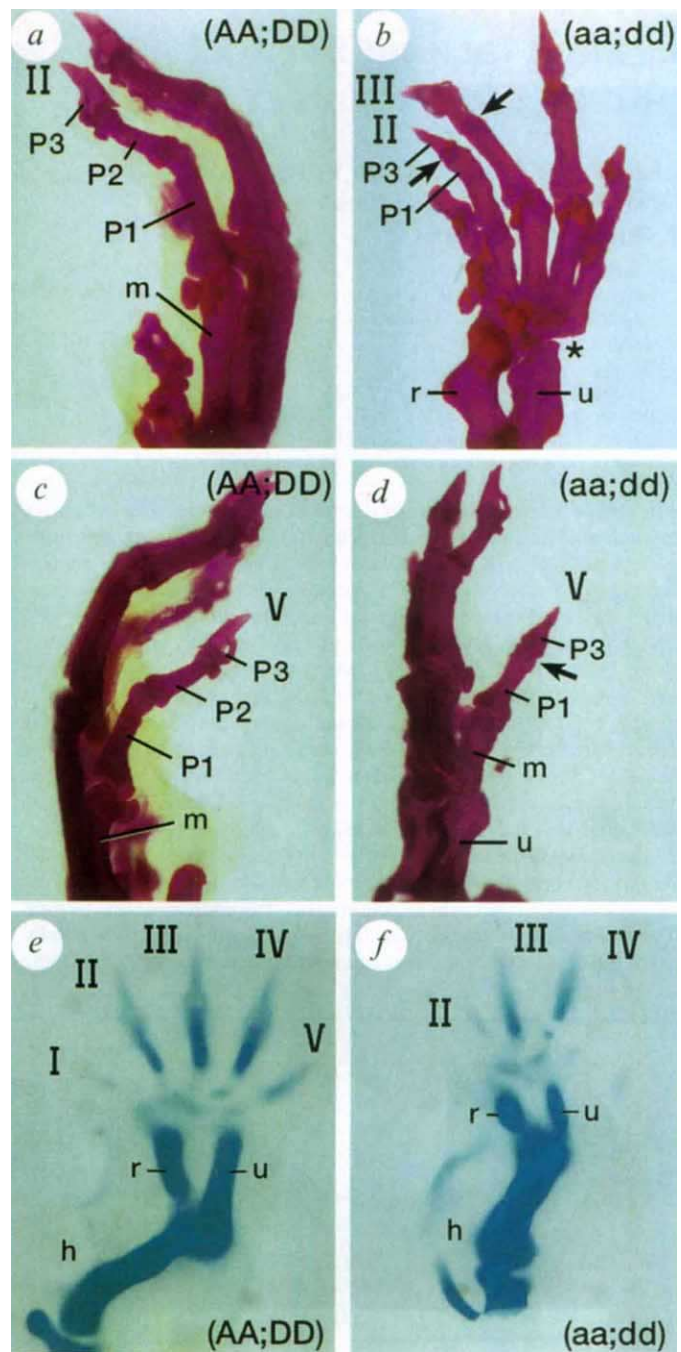


FIG. 2 Autopod defects in the double-mutant mouse. a, Wild-type (AA; DD) digit II with normal phalanges (P3, P2, P1) and metacarpal (m). b, The double mutant (aa; dd) shows several defects in the autopod. P2 is missing from digit II and is fused to P1 in digit III (arrows). The pisiform and triangular carpal bones are missing (star) and the navicular lunare is deformed. A small vestige of the radius (r) and ulna (u) remains. Digits I, II and III curve preaxially. Digit IV appears relatively normal. c, Wild-type (AA; DD) digit V with normal phalanges and metacarpal. d, The double mutant (aa; dd) digit V lacks P2 (arrow). e, Cartilage condensations in the developing right forelimb of wild-type mouse (AA; DD) at embryonic stage E13.5, showing the humerus (h), radius (r) and ulna (u) with digits I-V all initiated. f, Zeugopod defect is already evident in the double mutant (aa; dd) with a reduced radius and ulna. Only digits IV, III and part of II have started to condense. The scapula became detached from the mutant limb and is not shown. METHODS. Embryos were collected, genotyped and stained with Alcian blue as described¹⁹.

tions in the double mutant (*aa; dd*) paw are delayed with only digits IV, III and part of II formed.

Intermediate genotypes with three mutant alleles produce intermediate phenotypes in the radius and ulna. These animals have a reduction in the length of the zeugopod as well as a substantial bowing of the radius (Fig. 3*a-c*). As predicted from a quantitative phenotype, (*Aa; dd*) and (*aa; Dd*) limbs cannot be distinguished from each other by inspection. The hindlimbs of the double mutant are also affected (Fig. 3*d, e*). In double-mutant mice, the fibula is thickened and flared at its distal end and is never fused with the tibia, all in contrast to wild-type mice. Most noticeable, however, is the absence of the proximal tarsal bones (the talus and calcaneus/processus trochlearis) from double-mutant mice. This forces the tibia and fibula to join the foot at the mid-tarsals. Interestingly, the hindlimb phenotype is not quantitative with respect to the number of mutant alleles because intermediate genotypes (*aa; Dd*) and (*Aa; dd*) do not have intermediate phenotypes.

The normal mouse axial skeleton is characterized by 7 cervical (C1-7), 13 thoracic (T1-13), 6 lumbar (L1-6), 4 sacral (S1-4), and a variable number of caudal vertebrae. The transverse processes on S1-3 fuse to form the sacrum. Table 1 shows a summary of the vertebral phenotypes for mice generated from intercrosses of compound heterozygotes. Both the (*aa; DD*) and the (*AA; dd*) mutant mice show an apparent homeotic transformation of the first sacral vertebra towards a lumbar vertebra (S1→L)¹⁵⁻¹⁷. Half of the compound heterozygotes (*Aa; Dd*) show this same transformation, a phenotype never observed in either of the individual heterozygotes (*Aa; DD*) and (*AA; Dd*). Again, this quantitative phenotype suggests that only two mutant alleles (either of *hoxa-11*, or *hoxd-11*, or one from each locus) are necessary to induce this homeosis. The double mutant (*aa; dd*) has nine lumbar vertebrae (Table 1). These can be

accounted for by both a posterior homeotic transformation (T13→L1) and a double anterior homeotic transformation (S1, S2→L8, L9). In addition, the sacral vertebrae do not fuse to form a sacrum, and in some cases the second sacral vertebra and one caudal vertebra are deleted.

hoxa-11 and *hoxd-11* are expressed in the primitive blastemal cells that populate the nephrogenic zone of the developing kidney (ref. 18, and S.S.P., unpublished results). Whereas mice mutant for either *hoxa-11* or *hoxd-11* have normal kidneys^{15,16}, double mutants (*aa; dd*) suffer renal malformations that generally result in perinatal death (Fig. 4*a, b*). Some double-mutant mice are born lacking one or both kidneys. Newborn (*aa; dd*) mice with kidneys show severe renal hypoplasia (Fig. 4*b*), with poorly developed cortex and medulla and little or no nephrogenic activity in the subcapsular region (Fig. 4*c, d*). The rare adult (*aa; dd*) kidney has a thick wall of cortical tissue and poorly developed renal papillae. The renal tubules present are well developed and the glomerulae, although severely reduced in number, show only mild compensatory hypertrophy (results not shown). There were no abnormalities in the lower renal tract to suggest urinary obstruction. With rare exceptions, one wild-type allele of either *hoxa-11* or *hoxd-11* was sufficient for normal kidney development. Urogenital malformations also include homeotic transformation of the vas deferens towards an epididymis. This is apparent at the gross level, with the vas deferens of (*aa; dd*) mice being smaller in diameter and more coiled than normal (Fig. 4*e, f*), and also evident at the histological level, with the mutant vas deferens showing reduction of the muscle layer, lack of convolution of the epithelial layer, and an epididymis-like epithelial cell morphology with nuclei positioned peripherally instead of centrally (Fig. 4*g-i*).

We expect that quantitative interactions between *Hox* genes will be the rule rather than the exception. Previously we demon-

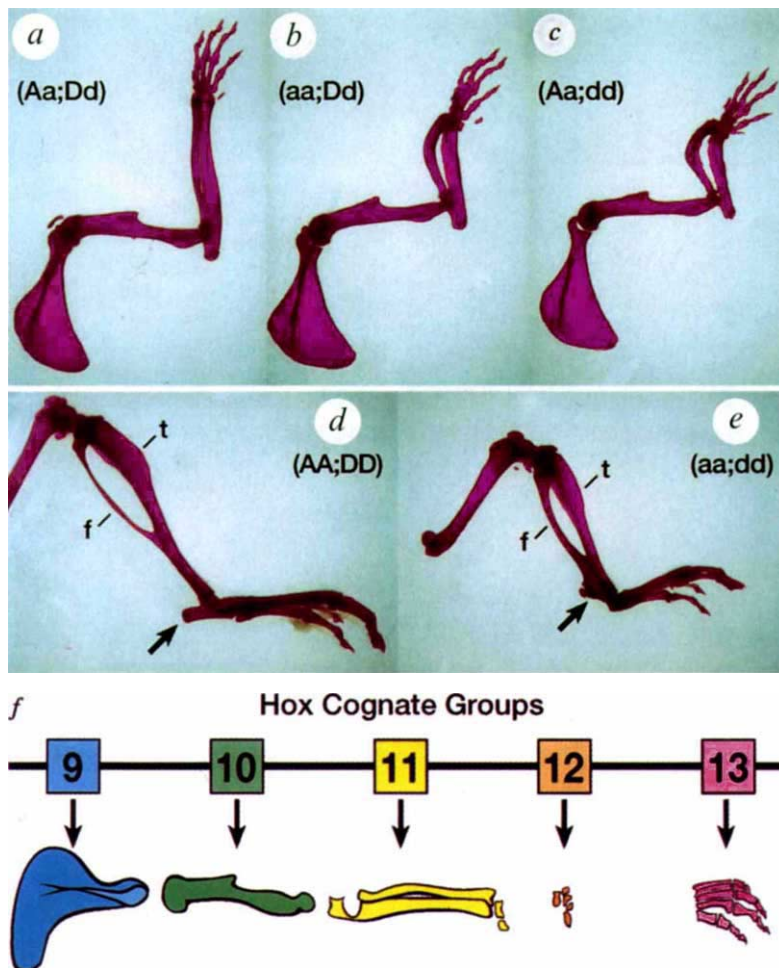


FIG. 3 Genetic quantification and synergy in the limb phenotypes. *a*, Compound heterozygote (*Aa; Dd*) forelimb. *b, c*, Mice with three mutant alleles (*b*, (*aa; Dd*) and *c*, (*Aa; dd*)) show intermediate phenotypes with a shortening and thickening of the zeugopod and with a substantial bowing of the radius. *d*, Wild-type (*AA; DD*) right hindlimb with normal tibia (*t*) and fibula (*f*) and proximal tarsal bones present (arrow). *e*, Double mutant (*aa; dd*) has malformed tibia and fibula that are slightly thicker and never fuse together, the proximal tarsal bones are also missing (arrow). *f*, A model for the role of *Hox* genes in limb development. The *Hox* cognate groups 9-13 represent the combined paralogous genes of the *Hox* loci and are drawn in the 3' to 5' direction. We propose that the cognate genes work in union to specify limb bone formation in the proximal to distal direction (left to right). In the double-mutant mouse (*aa; dd*), both *hoxa-11* and *hoxd-11* are mutated; this results in the loss of the radius, ulna and proximal carpals (yellow coded bones). In addition to these paralogous interactions, *Hox* genes in the same linkage group or across groups may also interact during limb development.

strated that the two paralogues *hoxa-3* and *hoxd-3* interact quantitatively to specify the craniocervical joint¹⁹ and that *hoxb-5* and *hoxb-6* function together to specify cervical and thoracic vertebrae²⁰. Here we describe quantitative, synergistic genetic interactions between *hoxa-11* and *hoxd-11*. In the vertebral column and carpal bones, compound heterozygotes have phenotypes similar to mice homozygous for mutations in either *hoxa-11* or *hoxd-11*. The quantitative effect is further revealed in animals with three mutant alleles: forelimbs in (aa; Dd) and (Aa; dd) mice show more severe defects in the zeugopod and autopod than are found in animals with only two mutant alleles. Synergy is seen in the axial column, limbs, kidneys and vas deferens, where phenotypes of the double mutant (aa; dd) are more pronounced than the sum of the phenotypes seen in the individual mutant homozygotes.

Limb bones are formed from prechondrogenic condensations that segment and bifurcate along the proximodistal axis to create the long bones (humerus, then radius and ulna). This is followed by proximodistal and anteroposterior condensations to generate the carpals, metacarpals and digits^{21,22}. Mice with targeted disruptions of either *hoxa-11* or *hoxd-11* show regional malformations in the shape, length and segmentations of bones in the zeugopod and autopod¹⁵⁻¹⁷. These defects do not resemble homeotic transformations, but rather perturbations in the growth of the cells contributing to the prechondrogenic condensations. Further, the defects are not polarized with respect to the anteroposterior axis. It is therefore surprising that in double mutants the radius and ulna are almost entirely eliminated. In

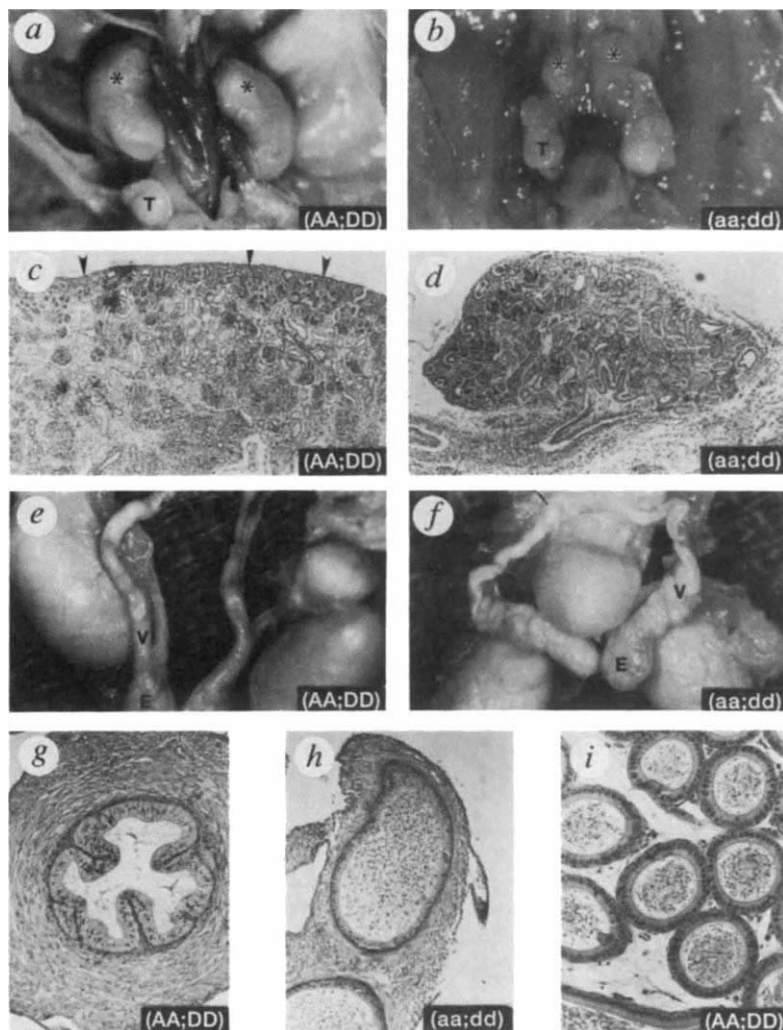
addition, the phenotype of the (aa; dd) autopod is strongly polarized. The more posterior proximal carpals are severely affected, whereas the distal carpals seem normal. The digits are also malformed. Digit IV seems to be normal, but the severity in phenotypes increases through digit III (a fusion between P2 and P1) to a maximum in digits II and V (both lacking P2). This phenotypic progression parallels the developmental 'limb plan' that proposes a basic axis of development that proceeds through the humerus, the ulna, and then anteriorly through the wrist such that the anterior distal carpals are the last to be made^{21,23}. These wrist cartilages then branch to form the digits in a specific temporal sequence: digits IV and III are the first to be made, followed by digits I, II and V²¹.

The limb phenotype of the double mutants (aa; dd) suggests that these two genes function together to specify the radius and the ulna. *Hox* genes are activated in the limb bud in a temporal sequence with the 3' genes expressed first and the 5' genes last, creating a transcriptional cascade of *Hox-9*, -10, -11, -12 and then -13 (ref. 11). We propose that it is the activation order and the interaction of the paralogues that specify patterns of prechondrogenic condensations along the proximodistal axis (Fig. 3f). Their role may be achieved by regulating cell proliferation and/or survival as well as specifying cell identity (that is, the number of chondrocytes). Thus the *Hox* group 11 genes would specify the zeugopod. In the double mutant mouse, *hoxa-11* and *hoxd-11* gene products are both absent, and this region is made as a rudiment at best. But once this crisis period is over, the *Hox* group 12 genes would be activated and limb develop-

FIG. 4 Urogenital defects in the double-mutant mouse.

a, Urinary tract of a wild-type newborn. The kidneys are indicated by the asterisk just above the testes (T). b, Urinary tract in the (aa; dd) mutant. The kidneys are extremely hypoplastic but the testes (T) are normal in size. c, Histology of wild-type kidney. Note the uniform zone of active nephrogenesis just beneath the renal capsule (arrowheads). The immature tubules and primitive glomeruli just beneath the capsule are separated by nests of undifferentiated blastemal cells. d, Histology of the (aa; dd) mutant kidney. In contrast with the wild-type kidney, the entire mutant kidney is easily seen at the same magnification. Although the tubules and glomeruli present are well developed, there is a decrease in total number of nephrons, with little nephrogenesis in the subcapsular zone. e, Dissected male reproductive tract from a wild-type mouse. There is a sharp transition from the epididymis (E) to the vas deferens (V). The vas deferens is a thick cord with a relatively straight configuration. f, Reproductive tract from the (aa; dd) mutant. The transition from the epididymis (E) to the vas deferens (V) is more obscure. The proximal vas deferens has a highly coiled configuration. g, Histology of the wild-type vas deferens. The epithelial layer has prominent folds and the lining cells have a large amount of cytoplasm with their nuclei in the middle or apical aspect of the cells. There is also a well developed smooth muscle layer in the wall of the vas deferens. h, Histology of the (aa; dd) mutant vas deferens. The epithelial lining is simple with no folds, and the cells are columnar and have nuclei with a uniform distribution in the basal aspects of the cells. The layer of smooth muscle cells in the wall is greatly reduced. i, Histology of a normal epididymis. The tubules are lined by a simple layer of columnar cells with nuclei that are uniformly arranged in the basal aspects of the epithelial cells. Note the close resemblance of this epithelial lining to that of the vas deferens from the double-mutant mice in h.

METHODS. Tissue samples were fixed in Bouin's solution and processed as described¹⁸. Sections were stained with haematoxylin and eosin.



ment resumed. In this sense, it is important to note that after the zeugopodal defect, limb development does proceed in the double-mutant mouse, albeit with some dysmorphology. But clearly the autopod is less affected than the zeugopod. The autopodal phenotypes distal to the major zeugopodal defects are likely to be the result of a coupling between proximodistal and anteroposterior patterning^{24–28}. This coupling may be genetic, with group 11 genes directly influencing the timing of activation of group 12 and group 13 genes. Alternatively, morphological coupling may account for these defects. The elimination of both *hoxa-11* and *hoxd-11* gene products may reduce the overall mesenchymal cell population available to form the zeugopod and posterior carpals. A deficiency of cells may also alter the timing ('heterochrony') of formation of the rest of the limb. The second crisis would therefore be revealed at the end of autopod formation, when cartilaginous elements are competing to recruit the few remaining cells. This heterochrony would explain the delay in digit condensation seen during embryogenesis of the double mutant and account for the more severe phalangeal defects seen in digits II and V, which are the last elements formed in the last digits specified.

It is interesting that in the double mutants the forelimbs are more affected than the hindlimbs. Also, whereas (aa; Dd) and (Aa; dd) mice show an intermediate forelimb phenotype, the presence of a third mutant allele does not alter the hindlimb phenotype beyond what is seen in (aa; DD) or (AA; dd) mice. These differences may be a consequence of a third paralogue, *hoxc-11*, which is expressed more strongly in the hindlimbs²⁹ and could provide a compensatory function. This hypothesis would predict that in (*hoxa-11*, *hoxc-11*, *hoxd-11*) triple mutants, the tibia and fibula would be completely eliminated and show quantitative dependence on the dosage of mutant alleles. Also, the last vestige of the forelimb radius and ulna observed in the double mutant would be predicted to be absent.

Our model is supported by the *hoxd-13* mutant mouse in which the mutant limb phenotype is restricted to the autopod¹³, and predicts that in a (*hoxa-13*, *hoxd-13*) double mutant the forepaw should be absent. *hoxd-12* has no paralogue in the *Hox A* family and may therefore act on its own (or in conjunction with *hoxc-12*) to influence formation of the carpals and tarsals. Alternatively, *hoxd-12* may recruit its closest *Hox A* paralogue (*hoxa-11* or *hoxa-13*) to help specify this region. Single-mutant mice for *hoxa-10*, *hoxc-10* and *hoxd-10* should show subtle defects in the humerus and femur, but double and triple mutants should affect strongly the entire stylopod and initiate a cascade of less severe phenotypes downstream through the zeugopod and autopod. Consistent with the model, individual *hoxd-10* and *hoxc-10* mutant homozygotes show mild malformations of the humerus and femur (E. M. Carpenter, J. M. Goddard and M.R.C., unpublished results; S. L. Hostikka and M.R.C., unpublished results). From this model, the appendicular ground state would be achieved in an animal lacking all of the *Hox 10–13* cognate genes. This should be a limbless mouse. □

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1. Capecchi, M. R. *Scient. Am.* **270**, 54–61 (1994).
2. Holland, P. W. H., Garcia-Fernandez, J., Williams, N. A. & Sidow, A. *Development* (Suppl.), 125–133 (1994).
3. Ruddle, F. H. et al. *A. Rev. Genet.* **28**, 423–442 (1994).
4. Dollé, P., Izpisua-Belmonte, J.-C., Falkenstein, H., Renucci, A. & Duboule, D. *Nature* **342**, 767–772 (1989).
5. Dollé, P., Izpisua-Belmonte, J.-C., Brown, J. M., Tickle, C. & Duboule, D. *Genes Dev.* **5**, 1767–1776 (1991).
6. Duboule, D. *Curr. Opin. Genet. Dev.* **1**, 211–216 (1991).
7. Izpisua-Belmonte, J.-C., Tickle, C., Dollé, P., Wolpert, L. & Duboule, D. *Nature* **350**, 585–589 (1991).
8. Izpisua-Belmonte, J.-C., Falkenstein, H., Dollé, P., Renucci, A. & Duboule, D. *EMBO J.* **10**, 2279–2289 (1991).
9. Yokouchi, Y., Sasaki, H. & Kuroiwa, A. *Nature* **353**, 443–445 (1991).
10. Duboule, D. *BioEssays* **14**, 375–384 (1992).
11. Izpisua-Belmonte, J.-C. & Duboule, D. *Dev. Biol.* **152**, 26–36 (1992).
12. Morgan, B. A., Izpisua-Belmonte, J.-C., Duboule, D. & Tabin, C. *Nature* **358**, 236–239 (1992).
13. Dollé, P. et al. *Cell* **75**, 431–441 (1993).
14. Haack, H. & Gruss, P. *Dev. Biol.* **157**, 410–422 (1993).

15. Small, K. M. & Potter, S. S. *Genes Dev.* **7**, 2318–2328 (1993).
16. Davis, A. P. & Capecchi, M. R. *Development* **120**, 2187–2198 (1994).
17. Favier, B., Le Meur, M., Chambon, P. & Dollé, P. *Proc. natn. Acad. Sci. U.S.A.* **92**, 310–314 (1994).
18. Li, H. M. H. et al. *Development* **121**, 1373–1385 (1995).
19. Condie, B. G. & Capecchi, M. R. *Nature* **370**, 304–307 (1994).
20. Rancourt, D. E., Tsuzuki, T. & Capecchi, M. R. *Genes Dev.* **9**, 108–122 (1995).
21. Shubin, N. H. & Alberch, P. *Evol. Biol.* **20**, 319–387 (1986).
22. Oster, G. F., Shubin, N., Murray, J. D. & Alberch, P. *Evolution* **42**, 862–884 (1988).
23. Duboule, D. *Science* **266**, 575–576 (1994).
24. Tabin, C. *Cell* **80**, 671–674 (1995).
25. Niswander, L., Tickle, C., Vogel, A., Booth, I. & Martin, G. R. *Cell* **75**, 579–587 (1993).
26. Fallon, J. F. et al. *Science* **264**, 104–107 (1994).
27. Niswander, L., Jeffrey, S., Martin, G. R. & Tickle, C. *Nature* **371**, 609–612 (1994).
28. Cohn, M. J., Izpisua-Belmonte, J.-C., Abud, H., Heath, J. K. & Tickle, C. *Cell* **80**, 739–746 (1995).
29. Peterson, R. L., Papenbrock, T., Davda, M. M. & Awgulewitsch, A. *Mech. Dev.* **47**, 253–260 (1994).

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Crucial role of the pre-T-cell receptor α gene in development of $\alpha\beta$ but not $\gamma\delta$ T cells

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In T-cell precursors, the T-cell-receptor β chain is expressed before the T-cell-receptor α chain^{1,2} and is sufficient to advance T-cell development in the absence of T-cell receptor α chains^{3–7}. In immature T cells, the T-cell-receptor β protein can form disulphide-linked heterodimers with the pre-T-cell-receptor α chain^{8,9} and associate with signal-transducing CD3 molecules⁵. The recently cloned pre-T-cell-receptor α gene encodes a transmembrane protein that is expressed in immature but not mature T cells^{9,10}. Here we show that $\alpha\beta$, but not $\gamma\delta$, cell development is severely hampered in pre-T-cell-receptor α -gene-deficient mice, which establishes a crucial role for the pre-T-cell receptor in early thymocyte development.

Intrathymic T-cell development proceeds from CD4⁺CD8⁺ precursors through CD4⁺CD8⁺ intermediates into CD4⁺CD8⁺ and CD4⁺CD8⁺ mature thymocytes^{3,11}. In rearrangement-deficient mice, thymocyte development is arrested at the CD4⁺CD8⁺CD25⁺ stage^{6,7,12}. Productive T-cell-receptor (TCR)- β transgenes can partly relieve the developmental block, allowing the accumulation of immature CD4⁺CD8⁺ thymocytes but not mature CD4⁺CD8⁺ and CD4⁺CD8⁺ T cells^{3–7} that require positive selection by TCR $\alpha\beta$ (ref. 13). In T-cell precursors the TCR- β chain forms disulphide-linked heterodimers with the pre-TCR α (pT α) chain, and can associate with signal-transducing CD3 molecules^{5,8,9}.

Here we report on the role of the TCR β -pT α heterodimer in development, based on experiments with pT α -deficient mice. These animals were generated by gene targeting in embryonic stem (ES) cells using a deletion-type targeting vector. On homologous recombination, this construct eliminated exons 3 and 4 of the pT α gene encoding the connecting peptide, which contains the cysteine required for heterodimer formation, the transmembrane region, the cytoplasmic tail and most of the 3' untranslated region (Fig. 1). Homologous recombination in ES cells and the absence of the deleted gene segment in pT α ^{−/−} mice was verified by Southern blotting with appropriate probes (Fig. 1, and results not shown). Offspring from intercrosses of pT α ^{+/−}

TABLE 1 Absolute number of thymocytes with different phenotypes

Phenotypes	$pT\alpha^+$ ($\times 10^{-6}$)	$pT\alpha^{-/-}$ ($\times 10^{-6}$)
$CD4^-8^-3^{low}25^+$	25.6	54.0
	22.0	40.4
$CD4^-8^-\delta^+$	12.4	55.1
	14.6	32.0
$CD4^+8^+\delta^+$	10.0	6.5
	14.5	11.0
$CD4^+8^+$	34.54	1.19
	34.62	1.13
$CD4^+8^+TCR\beta^{int.}$	14.50	0.24
	13.75	0.23
$CD4^+8^-TCR\beta^{high}$	3.51	0.13
	3.05	0.13
$CD4^-8^+TCR\beta^{high}$	0.81	0.09
	1.39	0.13

Numbers were obtained from two different mice of each genotype from a 5-day-old litter.

mice were killed, thymus and bone marrow removed, and single cell suspensions prepared and analysed by cytofluorometry. There were no significant differences between numbers of marrow cells from age-matched mice and thymocytes from $pT\alpha^{+/+}$ and $pT\alpha^{+/-}$ age-matched mice, whereas the number of thymocytes in $pT\alpha$ -deficient animals was reduced to less than 10%.

Figure 2 shows subsets from thymus of $pT\alpha^+$ and $pT\alpha^{-/-}$ mice only, because there was no difference in subsets of bone marrow and because lymphoid organs from $pT\alpha^{+/+}$ and $pT\alpha^{+/-}$ mice did not differ. Both $pT\alpha^+$ and $pT\alpha^{-/-}$ mice contain $CD4^-8^-$, $CD4^+8^+$ and single-positive $CD4^+8^-$ and $CD4^-8^+$ thymocytes, but cells with CD4 and CD8 co-receptors are proportionally under-represented in $pT\alpha^{-/-}$ mice whereas the proportion of $CD4^-8^-25^+$ cells is drastically increased (Fig. 2). Both types of mice contain CD4 and CD8 co-receptor expressing cells with low, intermediate and high levels of TCR- β chain on

TABLE 2 Subsets among $CD4^-8^-3^{low}$ thymocytes

Phenotypes	$pT\alpha^+$ (%)	$pT\alpha^{-/-}$ (%)
$CD44^+25^-$	12.1	11.0
$CD44^+25^+$	2.20	2.30
$CD44^-25^+$	40.7	86.6
$CD44^-25^-$	45.0	0.10

Percentages were calculated from the same litter as described in Fig. 2 and Table 1.

the cell surface. The fraction of $CD4^-8^-$ thymocytes with TCR δ chains on the cell surface was more prominent in $pT\alpha^{-/-}$ mice, and both TCR β and TCR δ chains were stoichiometrically associated with CD3 molecules. The data also show that the TCR β chains were associated with TCR α chains, as revealed by double-staining with TCR β and TCR Va2, 3.2, 8 and 11 antibodies (Fig. 2, lower right). From triple stainings with various antibodies listed in Fig. 2 and thymocyte numbers, we calculated the absolute number of cells belonging to various thymocyte subsets as shown in Table 1; the data show that the pre-TCR is not required to generate normal numbers of $CD4^-8^-3^{low}25^+$ precursors of $\alpha\beta$ T cells. In fact, their number is increased in $pT\alpha^{-/-}$ mice, probably because of the developmental arrest in these mice (see below). There is likewise no decrease in the number of $CD4^-8^-$ or $CD4$ and $CD8$ co-receptor expressing $\gamma\delta$ T cells (Table 1). Table 2 shows that among $CD4^-8^-3^{low}$ cells, $pT\alpha^{-/-}$ mice have normal or increased numbers of $CD44^+25^-$, $CD44^+25^+$ and $CD44^-25^+$ cells, but no $CD44^-25^-$ cells, which represent 45% in $pT\alpha^+$ mice. As most thymocyte expansion occurs in this subset¹¹, it is not surprising that the absolute number of $CD4^+8^+$ cells as well as mature TCR $\alpha\beta^{high}$ $CD4^+8^-$ and $CD4^-8^+$ cells is much lower in $pT\alpha^{-/-}$ than in $pT\alpha^+$ mice. Also, the proportion of TCR- $\alpha\beta$ -positive cells among $CD4^+8^+$ cells is lower in $pT\alpha^{-/-}$ than in $pT\alpha^+$ mice. Lymph-node cells from 28-day-old $pT\alpha^{-/-}$ mice contain mature single positive CD4 and CD8 cells representing about 5% of numbers found in $pT\alpha^+$ littermates (results not shown).

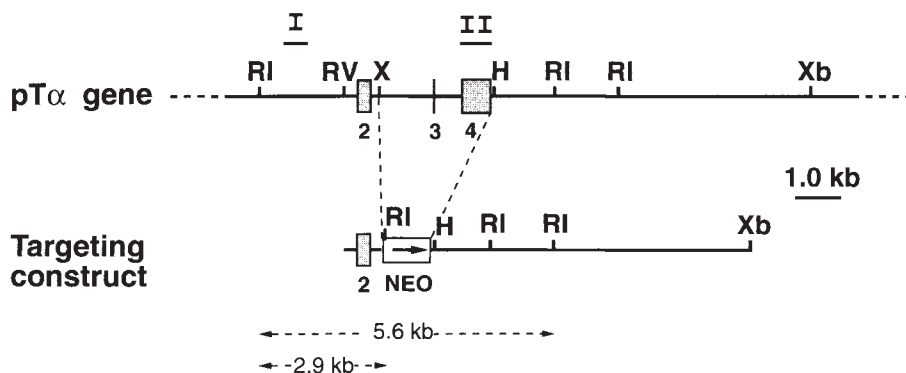


FIG. 1 Disruption of the $pT\alpha$ gene by homologous recombination: partial organization of the $pT\alpha$ locus²² and structure of the targeting vector. The $pT\alpha$ gene was cloned from a 129/Ola-derived genomic library (kind gift of A. Berns, Amsterdam). The targeting vector was constructed by replacing approximately 2.6 kilobases (kb) of the $pT\alpha$ sequence between the unique $XhoI$ site in intron 2 and a $BglII$ site ~60 base pairs (bp) upstream of the AATAAATAA polyadenylation site with a 1.2-kb $XhoI$ - $BamHI$ fragment of pMC1neoA (Stratagene) carrying the neomycin resistance gene (*neo*). The isogenic targeting construct was electroporated into E14.1 embryonic stem cells as described¹⁸. ES colonies surviving G418 selection were analysed by the polymerase chain reaction (PCR) in pools of 12 using primers specific for the *neo* cassette within the *tk* promoter (ATTGCGCAATGACAAGACGCTGC) and for the $pT\alpha$ gene just upstream of the $EcoRV$ site (GTTGGATGTTATTGGTTACTCTCTGA), respectively. Colonies within positive pools were re-screened individually by PCR and eventually by Southern analysis using

EcoRI-digested DNA and probe I (a 470-bp PCR fragment specific for $pT\alpha$ sequences outside the targeting construct, ~1.3 kb upstream of exon 2 (primers: TAGGTTTGAACACAGAT; TGATTTCTCTGTAGC)). Out of ~1,600 colonies screened, 3 had undergone homologous recombination, and one of these clones (pT355) gave rise to chimaeric mice. Chimaeric males were backcrossed with (C57BL/6 $\times$ DBA/2) F_1 females and heterozygous offspring carrying a mutant $pT\alpha$ allele were intercrossed to obtain mice deficient in $pT\alpha$. The absence of the deleted $pT\alpha$ sequences in homozygous knockout mice was confirmed by Southern blotting of *EcoRI*-digested genomic tail DNA and hybridization with a 310-bp *Apal*/*BspEI* complementary DNA fragment spanning the region of exon 4 that encodes the transmembrane portion, the cytoplasmic tail and 120 nucleotides of 3' untranslated sequence. Abbreviations for restriction sites: RI, *EcoRI*; RV, *EcoRV*; X, *XhoI*; H, *HindIII*; Xb, *XbaI*.

The above data indicate that pT α has no role in the development of most $\gamma\delta$ T cells for one of three reasons: (1) because it is not expressed in the $\gamma\delta$ lineage; (2) it cannot pair with the γ chain; or (3) because there is no need for a putative TCR γ -pT α heterodimer in $\gamma\delta$ T-cell development. Although this issue requires further investigation, we have been unable to detect RNA for pT α in thymocytes of TCR δ surface-positive thymocytes¹⁰. The increase in the number of $\gamma\delta$ cells could depend on the availability of space, and/or the possibility that ongoing $\gamma\delta$ rearrangement is not terminated by the pre-TCR. The fact that a few TCR $\alpha\beta$ -positive CD4/8-co-receptor-expressing thymocytes can be generated in the absence of the pre-TCR is consistent with an earlier finding of TCR α rearrange-

ments in TCR $\beta^{-/-}$ mice deficient in pre-T-cell-receptors⁶. The authors argued that CD4⁺8⁺ cells in TCR $\beta^{-/-}$ mice (which are often claimed erroneously not to contain CD4⁺8⁺ cells) could be of the δ lineage because they were absent in TCR $\beta^{-/-}$ $\times$ TCR $\delta^{-/-}$ mice. We find that in pT $\alpha^{-/-}$ mice CD4⁺8⁺3^{low}25⁺ precursors of $\alpha\beta$ T cells can differentiate, albeit inefficiently, into CD4⁺8⁺ cells with TCR $\alpha\beta$ on the surface. We therefore propose that the pre-TCR is sufficient and necessary for the generation of CD4⁺8⁺ precursors when no other TCR-expressing cells are present, and that in the presence of TCR-positive cells, the pre-TCR is required for the transition of CD4⁺8⁺25⁺ $\alpha\beta$ T-cell precursors, through rapidly dividing CD4⁺8⁺25⁺ cells into TCR $\alpha\beta$ -expressing CD4⁺8⁺ thymocytes.

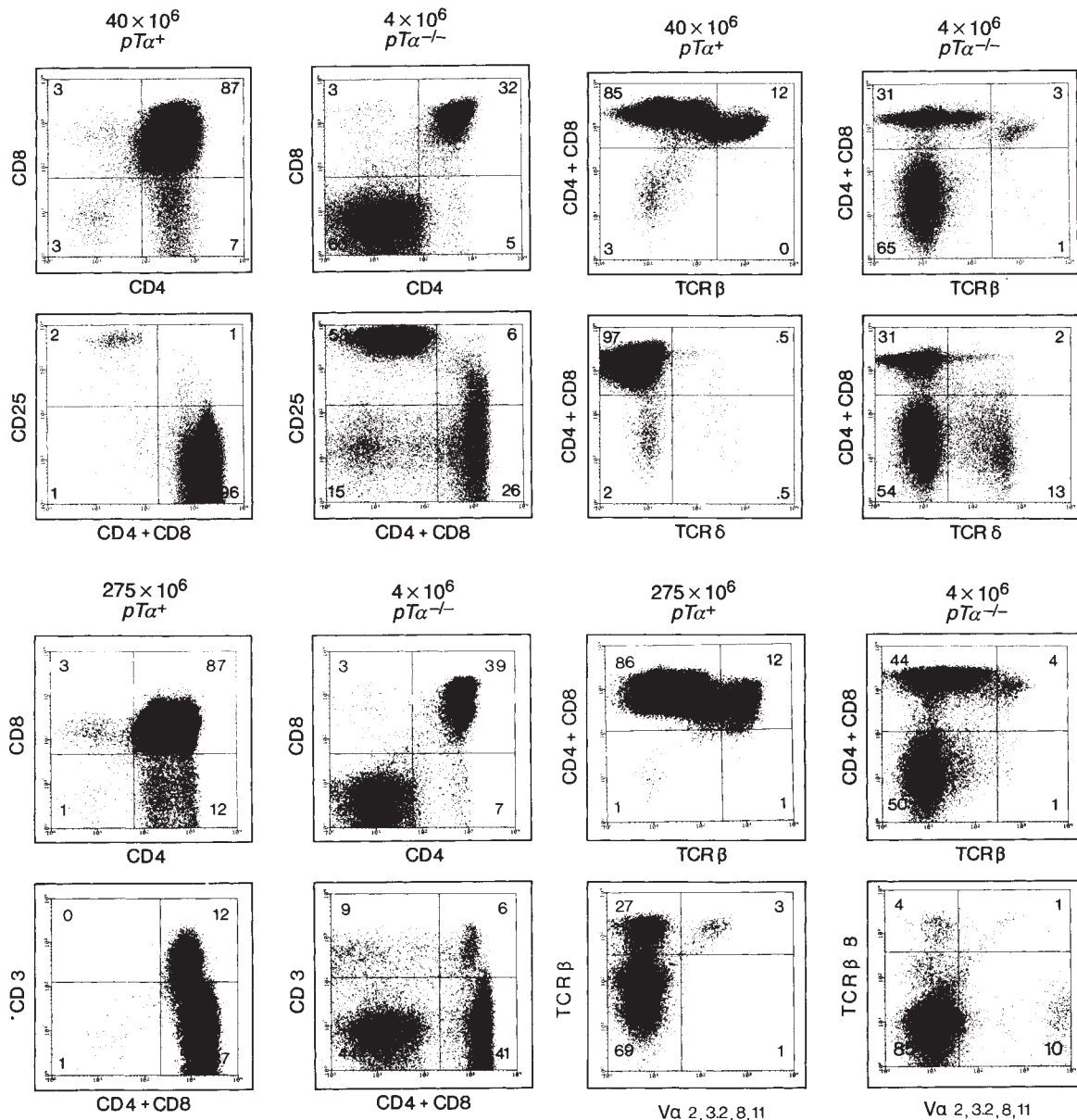


FIG. 2 Thymocyte subsets from pT α ⁺ and pT α ^{-/-} mice. Staining of thymocytes from 5-day-old (top two rows) and 28-day-old (bottom two rows) litters with CD4, CD8, TCR β , TCR δ and TCR-V α antibodies. All stainings were three-colour stainings with the following antibody-conjugates: anti-CD3-biotin and anti-CD3-FITC conjugates (500 A2 (ref. 19)), anti-TCR β -biotin and anti-TCR β -FITC (H57 (ref. 20)), anti-CD4-phycoerythrin (H129.19 Gibco), anti-CD8-biotin and anti-CD8-phycoerythrin (53-6.7 Pharmingen, San Diego), Anti-CD25-biotin (Pharmingen, San Diego), anti-CD44-phycoerythrin (ATCC collection KM81), anti-HSA-biotin (M1169 (ref. 21)), and anti-TCR δ -FITC (G3, Pharmingen, San

Diego). A cocktail of TCR-V α antibodies, namely anti-V α 2 (B20.1), anti-V α 3.2 (RR3.16), anti-V α 8 (KT50) and anti-V α 11 (RR8.1) was obtained from D. Mathis, Strasbourg. The biotin conjugates were revealed by streptavidin-Tricolor (Caltag, San Francisco). For each of the three colour stainings $\sim 4 \times 10^5$ cells were incubated with various reagents. In each case, 10^5 events were acquired for fluorescence-activated cell sorting by FACScan. The bright staining in the thymus (lower right) is due to thymic B cells that stain brightly with sheep anti-mouse Ig-FITC reagent, which was used to reveal the V α antibodies.

This view is consistent with data showing that TCR-positive thymocytes can induce the development of CD4⁺8⁺ cells when injected into rearrangement-deficient mice^{14,15} and explains the absence of CD4⁺8⁺ thymocytes in rearrangement-deficient^{6,7}, TCR-negative⁶ as well as CD3-negative mice (B. Malissen, personal communication). Differentiation through the CD4⁺8⁺25⁺ subset requires cell-autonomous signals delivered by the pre-TCR, and for that reason in normal mice almost all CD4⁺8⁺ T cells contain productive TCR- β genes¹⁶, whereas in the absence of the pre-TCR, only ~2% of TCR $\alpha\beta$ -positive CD4⁺8⁺ precursors are generated by some aberrant differentiation. Nevertheless, positive selection will operate on these cells and generate mature $\alpha\beta$ T cells whose number is regulated by homeostasis independent of the pre-TCR¹⁷. □

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1. Snodgrass, H. R., Dembic, Z., Steinmetz, M. & von Boehmer, H. *Nature* **315**, 232–233 (1985).
2. Raulet, D. H., Garman, R. D., Saito, H. & Tonegawa, S. *Nature* **314**, 103–107 (1985).
3. von Boehmer, H. A. *Rev. Immun.* **8**, 531–556 (1990).
4. Kishi, H. et al. *Embo J.* **10**, 93–100 (1991).

5. Groettrup, M., Baron, A., Griffiths, G., Palacios, R. & von Boehmer, H. *Embo J.* **11**, 2735–2745 (1992).
6. Mombaerts, P. et al. *Nature* **360**, 225–231 (1992).
7. Shinkai, Y. et al. *Science* **259**, 822–825 (1993).
8. Groettrup, M. et al. *Cell* **75**, 283–294 (1993).
9. Saint-Ruf, C. et al. *Science* **266**, 1208–1212 (1994).
10. Bruno, L. E. A., Rocha, B., Rolink, T., von Boehmer, H. & Rodewald, H. R. *Eur. J. Immun.* (in the press).
11. Kisielow, P. & von Boehmer, H. *Adv. Immun.* **58**, 87–209 (1995).
12. Rothenberg, E. V. *Adv. Immun.* **51**, 85–214 (1992).
13. Scott, B., Blüthmann, H., Teh, H. S. & von Boehmer, H. *Nature* **338**, 591–593 (1989).
14. Shores, E. W., Sharrow, S. O., Uppenkamp, I. & Singer, A. *Eur. J. Immun.* **20**, 69–77 (1990).
15. Lynch, F. & Shevach, E. M. *Int. Immun.* **5**, 991–995 (1993).
16. Dudley, E. C., Petrie, H. T., Shah, L. M., Owen, M. J. & Hayday, A. C. *Immunity* **1**, 83–93 (1994).
17. von Boehmer, H. & Hafen, K. *J. exp. Med.* **177**, 891–896 (1993).
18. Kuhn, R., Rajewsky, K. & Müller, W. *Science* **254**, 707–710 (1991).
19. Allison, J. P. in *UCLA Symposia on Molecular and Cellular Biology, New Series* (eds Kappler, J. & Davies, M.) (Lissabon, New York, 1987).
20. Kubo, R. T., Born, W., Kappler, J. W., Marrack, P. & Pigeon, M. J. *Immun.* **142**, 2736–2742 (1989).
21. Springer, T., Galfre, G., Secher, D. S. & Milstein, C. *Eur. J. Immun.* **8**, 539–551 (1978).
22. Fehling, H. J., Laplace, C., Mattei, M.-G., Saint-Ruf, C. & von Boehmer, H. *Immunogenetics* (in the press).

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The small heat-shock protein α B-crystallin as candidate autoantigen in multiple sclerosis

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THE identification of key antigens in human autoimmune diseases is a crucial step towards the development of specific intervention. The autoantigen(s) relevant to multiple sclerosis (MS) probably reside in myelin of the central nervous system, the target of the disease¹. Here we examine proliferative responses of human peripheral blood T cells to the complete collection of myelin proteins fractionated by reversed-phase high-performance liquid chromatography. Myelin isolated from MS-affected brain contained a single protein fraction to which T cells from MS patients and from healthy controls showed dominant responses. This highly immunogenic protein was identified as α B-crystallin, a small heat-shock protein. Immunohistochemical examination of MS lesions revealed the presence of oligodendrocytes and astrocytes with raised α B-crystallin expression, which were not found in unaffected myelin. Our findings indicate that α B-crystallin serves as immunodominant myelin antigen to human T cells when expressed at the elevated levels found in active MS lesions.

To examine responses of human T cells to the complete collection of myelin proteins from the central nervous system (CNS), short-term cultures were raised *in vitro* by priming peripheral blood mononuclear cells (PBMC) from 24 HLA-typed donors with total myelin protein. Proteins used for this purpose were

prepared from either healthy control myelin or from myelin purified from MS-affected white matter. Five PBMC donors were clinically defined MS patients and the other nineteen were healthy control subjects. After one cycle of restimulation with total myelin protein, T cells were collected and tested for proliferative responses to each of the forty reversed-phase high-performance liquid chromatography (RP-HPLC) fractions prepared from the same protein preparation as that used for priming. Figure 1a shows the RP-HPLC profile of fractionated total myelin protein from MS-affected brain, which is virtually identical to that from control material. Data from the proliferation assays were essentially the same in all cases, irrespective of HLA-type or clinical status of the donor. A representative set of data from five donors is shown in Fig. 1b.

The data in Fig. 1 illustrate two points. First, bulk T-cell responses after priming with all CNS myelin proteins were always directed primarily at the various minor myelin proteins contained in HPLC fractions 7–20. In contrast, responses to the major components proteolipid protein or myelin basic protein were barely significant. These findings extend our previous observations that the relative immunogenicity of individual CNS myelin proteins to human T cells bears no apparent relationship to their relative abundance in myelin². Second, the data in Fig. 1 reveal a distinct difference between responses to proteins derived from either healthy myelin or MS-affected tissue. In the latter case, a consistently predominant response was detected against the contents of RP-HPLC fraction 8. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) showed that this fraction contained only one detectable protein, with an apparent relative molecular mass (M_r) of about 23K (Fig. 2). The same protein was detected as the single component in the corresponding fraction derived from the control preparation but in smaller amounts.

To identify the 23K protein in fraction 8, the protein was purified from MS-affected white matter by RP-HPLC. Direct amino-acid sequencing of the purified preparation was unproductive, indicative of an N-terminal modification. Thus, the 23K protein was trypsinized and a selection of the resulting fragments was purified by RP-HPLC and sequenced. As a result, four internal sequences were identified that were identical to sequences of human α B-crystallin. The amino-acid composition of the purified 23K protein was consistent with the full sequence of human α B-crystallin. In line with this the behaviour of purified human 23K protein and bovine α B-crystallin was identical in RP-HPLC and in SDS-PAGE. Furthermore, polyclonal rabbit antibodies

raised against the purified 23K human protein were crossreactive with bovine α B-crystallin upon western blotting (Fig. 2).

To verify that α B-crystallin is indeed the immunogenic component in HPLC fraction 8, responses of myelin-primed T cells to fraction 8 were compared with those against α B-crystallin purified from bovine eye lens, which is identical to the human homologue at all but four of the 175 amino-acid positions. Figure 3 illustrates that responses of myelin-primed T cells to HPLC fraction 8 and purified α B-crystallin were comparable in proliferation as well as in their release of the pro-inflammatory cytokines interferon- γ and interleukin-2. Conversely, bulk T-cell cultures primed with purified bovine α B-crystallin were strongly crossreactive to total myelin protein as well as to the contents of HPLC fraction 8. Earlier reports identifying α B-crystallin as a small heat-shock protein^{3,4} prompted us to test whether

quantitative differences in the local expression of α B-crystallin could explain our findings (Fig. 1b). Expression of α B-crystallin was examined by immunohistochemistry of 34 MS plaques corresponding to different developmental stages from 28 MS patients and five control white-matter samples with no neuropathological condition. In all but two inactive plaques, α B-crystallin-expressing glial cells were found grouped within or at the edge of the lesional area. No, or very few, glial cells with α B-crystallin expression could be detected in control white matter or unaffected white matter from MS brains (data not shown). To identify the cellular origin of α B-crystallin in MS lesions, double staining was performed with myelin/oligodendrocyte glycoprotein and myelin-associated glycoprotein as a marker for oligodendrocytes, and glial fibrillary acidic protein as a marker for astrocytes (Fig. 4)^{5,6}. α B-crystallin colocalized with oligoden-

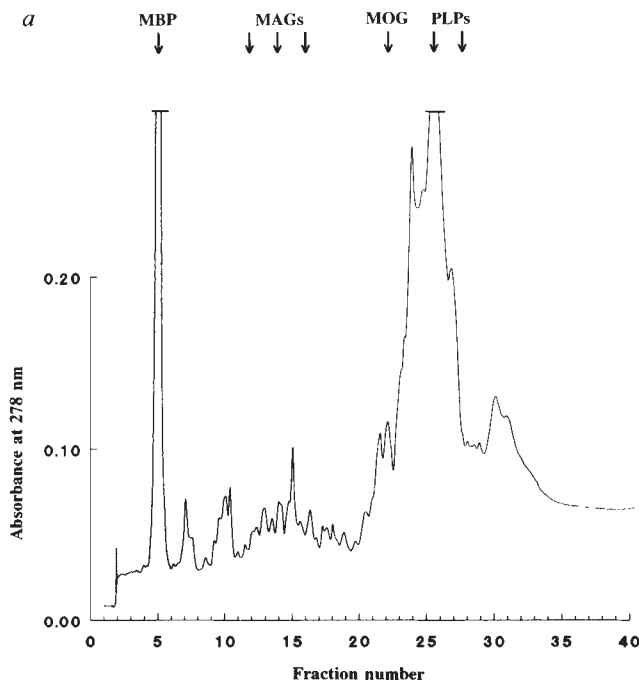
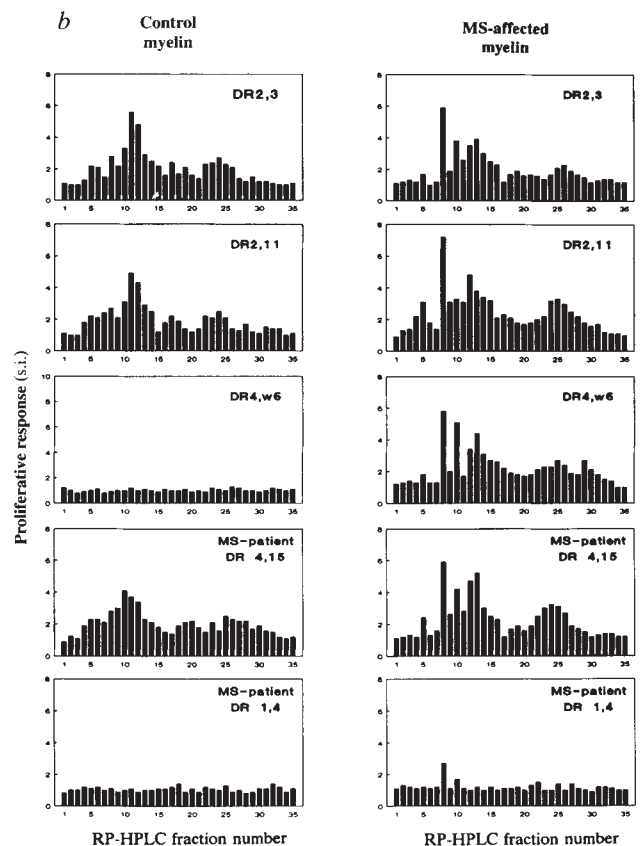


FIG. 1 a, RP-HPLC fractionation of the complete collection of CNS myelin proteins derived from MS-affected white matter²⁰. As previously shown, many myelin proteins elute as sharp single protein peaks, like myelin basic protein (MBP) (fractions 5/6) and myelin/oligodendrocyte glycoprotein (MOG) (fraction 22). Others, such as proteolipid protein (PLP) or myelin-associated glycoprotein (MAG), exist as differentially modified species with varying hydrophobicities and elute over a series of adjacent fractions. In this way, MAG is recovered in fractions 10–15 and PLP in fractions 22–32. b, Proliferative responses of myelin-primed T-cell cultures to HPLC-fractionated CNS myelin proteins from either control myelin (left panels) or MS-affected myelin (right panels). s.i., Stimulation index, defined as (response with antigen)/(response without antigen).

METHODS. CNS myelin was purified by density-gradient centrifugation²¹ from human white matter and collected as two distinct pools of samples. One pool contained myelin from eleven clinically definite MS patients; all samples include MS plaques, as confirmed by magnetic resonance imaging and histopathological examination. Control myelin was prepared from white matter from 21 control brains. Total myelin protein was extracted and delipidated as described²⁰. Before using the total protein extract as antigen, lyophilized protein was dissolved into 2-chloroethanol/0.1% trifluoroacetic acid and dialysed extensively against water. Protein concentrations were determined by amino-acid analysis and equal amounts of proteins derived from either myelin preparation were used throughout all experiments. Peripheral blood mononuclear cells (PBMC) from each donor were cultured in RPMI1640 (Dutch modification; supplied with 100 U ml⁻¹ penicillin, 0.1 mg ml⁻¹ streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES pH 7.4 and 10% pooled human serum) at 2×10^5 cells per 200 μ l at 37 °C in a humidified stove containing 5% CO₂. Total myelin protein



was added as priming antigen at 25 μ g ml⁻¹. After 7 days, growing T cells were restimulated with 10⁵ irradiated (30 Gy) autologous PBMC per 200 μ l and fresh antigen (25 μ g ml⁻¹). After another 4 days, recombinant human IL-2 was added to a final concentration of 50 U ml⁻¹. At day 14, T cells were collected, pooled and tested for proliferative responses to various antigens by seeding 5×10^4 T cells together with 5×10^4 irradiated autologous PBMC and antigen in 200 μ l of fresh culture medium. After 3 days, [³H]thymidine was added (20 kBq per well) and after another 24 h of culture, thymidine incorporation was determined using a β -plate counter. For use as a test antigen, HPLC-fractionated proteins were lyophilized, redissolved in 2-chloroethanol/0.1% trifluoroacetic acid and dialysed against water. Protein concentrations were determined by amino-acid analysis. A fixed proportion of the contents of each fraction was added as antigen to give a final protein concentration of ~ 50 μ g ml⁻¹ for the fractions containing the largest amounts of protein (fractions 5/6 and 24–27). Varying the dose of antigen yielded data consistent with the altered dose, indicating that the low responses to MBP or PLP were not due to inappropriate dosage (data not shown).

droglial as well as with astroglial markers in both acute and chronic MS plaques. Intense staining of oligodendrocytes for α B-crystallin was frequent in early lesions whereas astrocytic staining predominated in older lesions. Some staining of myelin itself was also distinguishable, but was close to background. There was no colocalization of α B-crystallin with markers specific for infiltrated T cells, B cells or macrophages. As previously reported⁷⁻¹⁰, enhanced α B-crystallin expression in some glial cells was also evident in white matter samples from donors

affected by other neuropathological diseases such as Alzheimer's, Parkinson's, Huntington's, Pick's and Lewy body diseases (data not shown), but staining of glial cells was generally less intense than in MS lesions and was lost more rapidly at higher serum dilutions.

Other heat-shock proteins, including cognates of the Hsp60, Hsp70 and Hsp90 families, have been detected in glial cells in MS lesions¹¹⁻¹³, but the factors inducing these larger heat-shock proteins are probably different from those that trigger expression

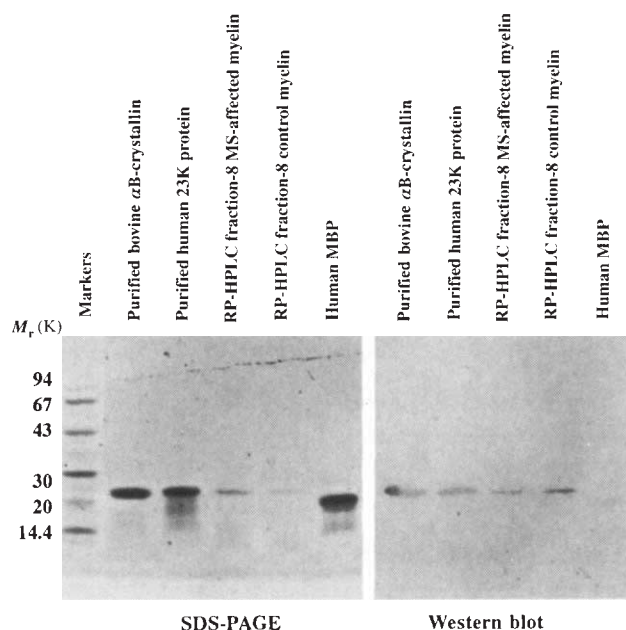


FIG. 2 SDS-PAGE and western blot analysis of the contents of HPLC fractions 8, compared to bovine α B-crystallin, purified 23K protein and human MBP, showing comigration between purified bovine α B-crystallin and the 23K protein contained in HPLC fractions 8. Western blotting reveals crossreactivity of a polyclonal rabbit serum raised against purified 23K protein towards bovine α B-crystallin. Sequence analysis of tryptic peptides from the 23K protein yielded the following sequences: LFDQFR (human α B-crystallin residues 23-28), YLR (residues 48-50), APSWFDLTGLSEMR (residues 57-69) and IPADVDP(L) (residues 124-131).

METHODS. Bovine α B-crystallin was purified by RP-HPLC from a commercial preparation of α -crystallin (Sigma). Standard SDS-PAGE analysis was done using an 8-25% gradient polyacrylamide gel (Pharmacia LKB). Equal samples of the contents of HPLC fraction 8 derived from either control myelin or MS-affected myelin were used for comparison. For western blotting a 1:25 dilution of rabbit serum raised against purified 23K protein from MS-affected myelin was used.

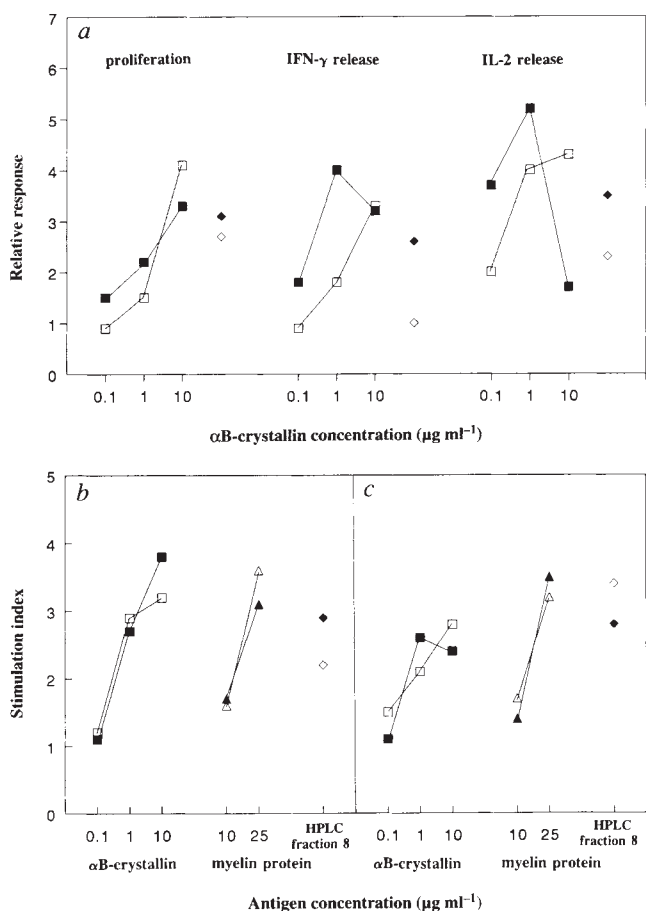


FIG. 3 Human T cells show full crossreactivity between total myelin protein, the contents of HPLC fraction 8, and α B-crystallin purified from bovine eye lens, identifying α B-crystallin as the immunogenic component in HPLC fraction 8. **a**, T cells primed with total myelin protein respond to purified α B-crystallin equally as well as they do to the contents of HPLC fraction 8. T cells from two donors (open symbols: DR2,4; closed symbols: MS patient DR4,15) were primed against total myelin protein. After 2 weeks, bulk responses against the contents of HPLC fraction 8 derived from MS-affected myelin (diamonds) were compared to those against varying amounts of purified α B-crystallin (squares). Proliferative responses as well as release of the pro-inflammatory cytokines IFN- γ and IL-2 are expressed relative to the levels found in the absence of any antigen. **b**, T cells primed against α B-crystallin cross-react with total myelin protein and the contents of HPLC fraction 8. **c**, T cells primed with the contents of HPLC fraction 8 crossreact with both α B-crystallin and total myelin protein. For **b** and **c**, PBMC were used from two other donors (open symbols: MS patients DR2,4; closed symbols: control donor DR 11,14). PBMC were primed with purified α B-crystallin at 1 μ g ml⁻¹ or with the contents of HPLC fraction 8 according to the protocol described in the legend to Fig. 2. After two weeks of culture, T cells were collected and assayed for proliferative responses to the antigens indicated. At the dosage of fraction 8 used in all experiments, the concentration of α B-crystallin as a component of this fraction was estimated by SDS-PAGE and western blotting at $\sim$ 1 μ g ml⁻¹.

METHODS. T cell priming and assays for proliferative responses are described in Fig. 2 legend. Release of IFN- γ and IL-2 were determined using culture supernatants drawn following a 2-day culture with the antigen indicated. Concentrations of IFN- γ were determined by ELISA (Central Laboratory of the Blood Transfusion Service, Amsterdam) and of IL-2 using the CTLL-L assay. Briefly, 5×10^3 CTLL-L cells were exposed to culture supernatant for 24 h before being pulsed with 20 kBq [³H]thymidine. Thymidine incorporation was determined as for Fig. 2.

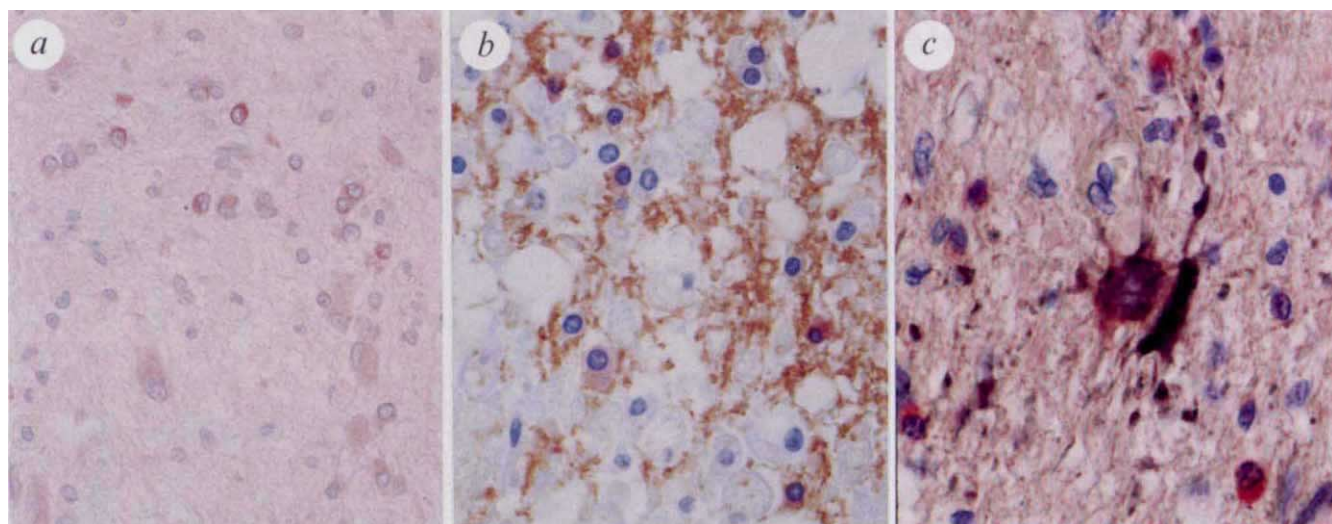


FIG. 4 Both oligodendrocytes and astrocytes in MS lesions show enhanced expression of α B-crystallin. Sections of formalin-fixed paraffin-embedded white-matter samples from active MS lesions were stained for human α B-crystallin, MOG as a surface marker for oligodendrocytes, or glial fibrillary acidic protein (GFAP) as a marker for astrocytes. **a**, Single staining of an acute MS lesion for α B-crystallin (red) showing high intracellular expression in some oligodendrocytes and moderate expression in astrocytes ($100\times$). **b**, Double staining for α B-crystallin (red) and MOG (brown) identifying oligodendrocytes as α B-crystallin-producing cells in active MS lesions ($133\times$). **c**, Double staining for α B-crystallin (red) and GFAP (dark blue) revealing α B-crystallin expression also in astrocytes ($250\times$). A double-stained astrocyte is seen surrounded by single-stained oligodendrocytes.

METHODS. Formalin-fixed paraffin-embedded human white-matter sections ($5\mu\text{m}$) were deparaffinized in xylene and hydrated in ethanol. Following blocking of endogenous peroxidase activity and a 10-min incubation in 10% FCS, sections were stained for the different markers.

Staining for α B-crystallin was done with a 1:1,500 dilution in 10% (vol/vol) FCS of polyclonal rabbit antibodies raised against α B-crystallin purified from MS-affected human myelin. After rinsing, binding was visualized using biotinylated donkey anti-rabbit antibodies and, subsequently, avidin-labelled alkaline phosphatase and fast red (Sigma). Specificity of the α B-crystallin staining was confirmed by the observation that preincubating polyclonal rabbit antiserum with RP-HPLC-purified bovine α B-crystallin from eye lens at $5\mu\text{g ml}^{-1}$ completely blocked the staining observed. No blocking was observed when the serum was preincubated with a $5\mu\text{g ml}^{-1}$ purified bovine α A-crystallin, which shares about 60% sequence identity with α B-crystallin. MOG staining was performed with a murine anti-MOG monoclonal antibody, followed by peroxidase-conjugated goat anti-mouse antibody (Dianova Jackson) and 3'-diaminobenzidine. GFAP was visualized using a murine monoclonal antibody against GFAP (Boehringer Mannheim); secondary steps were the same as for anti-MOG staining except for the use of chloronaphthol as a chromogen. Counterstaining was performed using haematoxylin.

of the small Hsp α B-crystallin^{14,15}. Also, rather than triggering responses in T cells with $\alpha\beta$ T-cell receptors, Hsp60 and Hsp70 may help mount responses by $\gamma\delta$ -bearing T cells^{11,13}. Flow cytometry of short-term T-cell cultures raised against α B-crystallin provided no evidence for recruitment of $\gamma\delta$ T cells by α B-crystallin *in vitro* (data not shown).

Our key finding is that α B-crystallin acts as immunodominant myelin antigen to human T cells when expressed at the increased levels found within and immediately around active MS lesions in the human brain. Stress-induced expression of α B-crystallin in human disease has so far been found primarily in CNS glial cells and much less—if at all—in other types of cells or tissues^{7,10,16}. Neurodegenerative diseases, neurotropic infection and oncogene expression are among the factors found so far to be associated with elevated α B-crystallin expression in the CNS. Constitutive expression occurs in several other tissues, including eye lens, cardiac muscle and kidney epithelial cells^{7,17,18}. Together, these data indicate that the expression of α B-crystallin is unlikely on its own to trigger demyelinating autoimmunity in MS, but may contribute to the amplification of local inflammatory responses in parallel with disturbance of the blood-brain barrier, cytokine production and expression of major histocompatibility antigens, and of co-stimulatory and adhesion molecules. Our data indicate that peripheral blood T cells from MS patients and healthy controls respond similarly to α B-crystallin, as they do to other self antigens^{1,19}, which suggests that local antigen-presenting features of the target tissue itself, rather than any aberrance of the peripheral T-cell repertoire, are crucial to the development of autoimmunity in MS. The fact that the immunodominant myelin antigen identified here is expressed at locally regulated levels is in line with this view. MS lesions may

develop if pro-inflammatory factors—including the autoantigen itself—accumulate locally beyond a threshold of control as a result of stress-producing events such as local immune responses to viral antigens. We propose that α B-crystallin in this context serves as key myelin antigen in the development of MS. □

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- Martin, R., McFarland, H. F. & McFarland, D. E. *Rev. Immun.* **10**, 153–187 (1992).
- Van Noort, J. M. et al. *J. Neuroimmun.* **46**, 67–72 (1993).
- Ingolia, T. D. & Craig, E. A. *Proc. natn. Acad. Sci. U.S.A.* **79**, 2360–2364 (1982).
- Klemenz, R., Fröhli, E., Steiger, R. H., Schäfer, R. & Aoyama, A. *Proc. natn. Acad. Sci. U.S.A.* **88**, 3652–3656 (1991).
- Ozawa, K. et al. *Brain* **117**, 1311–1322 (1994).
- Brück, W. et al. *Ann. Neurol.* **35**, 65–73 (1994).
- Iwaki, T., Kume-Iwaki, A., Liem, R. K. H. & Goldman, J. E. *Cell* **57**, 71–78 (1989).
- Head, M. W., Corbin, E. & Goldman, J. E. *J. Cell. Physiol.* **159**, 41–50 (1994).
- Iwaki, T. et al. *Am. J. Path.* **140**, 345–356 (1992).
- Lowe, J. et al. *J. Path.* **166**, 61–68 (1992).
- Selmaj, K., Brosnan, C. F. & Raine, C. S. *Proc. natn. Acad. Sci. U.S.A.* **88**, 6452–6456 (1991).
- Wucherpfennig, K. W. et al. *Proc. natn. Acad. Sci. U.S.A.* **89**, 4588–4592 (1992).
- Salveti, M. et al. *J. Autoimmun.* **5**, 691–702 (1992).
- Karlik, C. C., Couto, M. D. & Fryberg, E. A. *Cell* **38**, 711–719 (1984).
- Hiromi, Y. & Hotta, Y. *EMBO J.* **4**, 1681–1687 (1985).
- De Jong, W. W., Lubsen, N. H. & Kraft, H. J. *Prog. Ret. Eye Res.* **13**, 391–442 (1994).
- Kato, K. et al. *J. Biol. Chem.* **267**, 7718–7725 (1992).
- Zantema, A., Verlaan-De Vries, M., Maasdam, D., Bol, S. & van der Eb, A. J. *J. Biol. Chem.* **267**, 12936–12941 (1992).
- Pette, M. et al. *Neurology* **40**, 1770–1778 (1990).
- Van Noort, J. M., El Ouagmiri, M., Boon, J. & Van Sechel, A. C. *J. Chromat.* **653**, 155–161 (1994).
- Norton, W. T. & Poduslo, S. E. *J. Neurochem.* **31**, 749–757 (1973).

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Mediation by HLA-DM of dissociation of peptides from HLA-DR

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HUMAN leukocyte antigen (HLA)-DM is an unconventional major histocompatibility complex (MHC) class II heterodimer that is important for B-cell-mediated antigen processing and presentation to MHC class II-restricted T cells¹⁻¹². HLA-DM is encoded by two genes, *DMA* and *DMB*, which map to the MHC class II region¹, and shares some homology with MHC class I and class II proteins^{2,3}. Here we define the biochemical role of HLA-DM. Recombinant soluble HLA-DM heterodimers have been purified from culture supernatants of insect cell transformants. At pH 5.0, they induce the dissociation of a subset of peptides bound to HLA-DR, including a nested set of class-II-associated invariant chain peptides (CLIP). This process liberates HLA-DR and leads to the enhanced binding of exogenous peptides.

Human B-cell lines deficient in HLA-DM are defective in MHC class II-restricted antigen presentation. Although the surface level of MHC class II on DM mutant cells is normal, and these molecules can be loaded with exogenously supplied peptides, B cells lacking DM are inefficient in the processing and presentation of certain protein antigens⁵⁻⁸. Furthermore, MHC class II molecules expressed in these B-cell lines are biochemically distinct in that they lack certain conformation-dependent antibody epitopes⁸, are unstable on SDS-PAGE (polyacrylamide gel electrophoresis) gels^{7,8}, and are filled almost exclusively with CLIP^{4,8,9}. Replacement of CLIP with cognate peptides stabilizes MHC class II dimers in these cells^{4,10}. Restoration of DM expression re-establishes the normal phenotype of the MHC class II molecules, as well as the ability of the cells to process and present antigens normally^{2,11,12}. Anti-DM antisera detect the presence of DM within an intracellular compartment that is a putative site of peptide loading onto MHC class II molecules^{2,3,13-18}. These data demonstrate that DM is important for the intracellular loading of peptides onto MHC class II molecules, but the precise function of DM remains unknown.

To facilitate the functional characterization of DM molecules we have generated stable transformants of *Drosophila* S2 cells which secrete a soluble form of DM (sDM) in which the transmembrane and cytoplasmic domains of DM- α and DM- β are truncated and replaced with FLAG¹⁹ and simian virus 40 (SV40) large T-antigen²⁰ epitope tags, respectively. Expression of the recombinant protein was assessed by western blot analysis of culture supernatants. S2 cells expressing sDM- α secreted protein of relative molecular mass (M_r) 25K when analysed by SDS-PAGE (Fig. 1a). S2 cells expressing sDM- β secreted protein of M_r 25K under reducing conditions, but aggregates of high M_r otherwise (Fig. 1a). Coexpression of sDM- α and sDM- β partly prevented the aggregation of sDM- β on non-reducing gels, indicating that the sDM- α and sDM- β chains were interacting (Fig. 1a). This was confirmed by SDS-free non-denaturing PAGE analysis, which demonstrated interchain pairing between the secreted sDM- α and sDM- β proteins (Fig. 1b). Secreted sDM heterodimers were purified using anti-FLAG affinity columns followed by gel filtration chromatography (Fig. 1c-e).

The influence of sDM on peptide binding to DR was evaluated by performing *in vitro* binding assays. The DR molecules used in these assays were purified by affinity chromatography from five different sources: DRB1*0101 (DR1), DRB1*0301 (DR3) and DRB1*0401 (DR4) from lysates of human B-cell lines, and soluble recombinant DRB1*0101 (sDR1) and DRB1*0401 (sDR4) from supernatants of S2 cell transformants. The sDR1 and sDR4 molecules produced by S2 transformants have properties similar to sDR1 produced in a baculovirus system²¹. In all cases, the addition of sDM to the binding assay enhanced the amount of peptide bound by DR (Fig. 2a). Enhanced peptide binding was not seen on addition of the control proteins H-2/I-A^d, immunoglobulin and albumin. sDM had no effect on the non-binding control peptide ovalbumin (323-339) (data not shown). The 50% effective doses for peptides binding to DR were not altered by the presence of sDM (Fig. 2b). Thus sDM does not affect peptide affinity for DR but appears to increase the availability of DR for binding peptides.

To examine the influence of sDM on peptide loading to DR in more detail, the association rate of the binding of three different peptides to sDR1 was measured in the presence and absence of sDM (Fig. 2c). The apparent on-rate of all three peptides was enhanced by sDM to a similar degree. However, because the rate of peptide binding to DR may be limited by the dissociation rates of prebound peptides, we explored the possibility that sDM might exert its effects by accelerating the dissociation of these peptides. Dissociation rates from sDR1 were measured in the presence or absence of sDM (Fig. 2d). The off-rates of CLIP (80-103) and myelin basic protein (90-102) were dramatically increased by sDM, but there was no effect on the off-rate of haemagglutinin (307-319). Thus the interaction of sDM with DR can lead to the enhancement release of some, but not all, bound peptides. A requirement for DM to enhance the release of CLIP from DR would account for the predominant association of these peptides with DR isolated from DM mutant cell lines. However, the sDM-mediated release of peptides from DR is not restricted to CLIP (Fig. 2d and data not shown). Indeed, sDM can enhance peptide binding to sDR derived from S2 transformants not expressing invariant chain (Fig. 2a, b).

DM-mediated enhancement of peptide binding to sDR1 and sDR4 was optimal at a pH range of 4.5 to 5.5 (Fig. 3a), which corresponds to that of the endosomal compartment where DM and DR are colocalized¹⁴. In addition, DM accelerated the rate of peptide dissociation from sDR1 and sDR4 at pH 5.0, but not at pH 7.0 (Fig. 3b).

We have previously described a B-cell line expressing a mutant DR3 in which a proline-to-serine substitution at position 96 of the DR- α chain (DR3-Ser 96) results in the formation of an extra N-linked glycosylation site²². The mutant DR3 had similar characteristics to that of wild-type DR3 expressed in DM-deficient B-cell lines, suggesting that the DR3 structural mutation might prevent interaction of DR3 with DM. To test this hypothesis, we compared the ability of sDM to enhance the binding of peptides to DR3 isolated from four different sources: wild-type B cells, DM mutant B cells, B cells expressing DR3-Ser 96, and B cells expressing DR3 with a proline-to-alanine substitution at position 96 of the DR- α chain (DR3-Ala 96) (Fig. 4a). B cells expressing DR3-Ala 96 have an unaltered DR3 glycosylation pattern and a normal phenotype (C. Bergman, T. Saweda, M.-d. Y. Chang and E. Mellins, submitted). DR3 from DM-mutant cells and DR3-Ser 96 bound more peptide than wild-type DR3 and DR3-Ala 96, even in the absence of sDM. This is presumably because most of these DR3 molecules are loaded with peptides that are more readily exchangeable during the time course of the *in vitro* binding assays. For all peptides tested, an enhanced binding was observed upon the addition of sDM to wild-type DR3, DR3 from DM-mutant cells, and DR3-Ala 96, but not DR3-Ser 96. Furthermore, the addition of sDM accelerated the dissociation of CLIP (80-103) from wild-type DR3, DR3 from DM-mutant cells, and DR3-Ala 96, but not

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from DR3-Ser 96 (Fig. 4b). These data demonstrate that the additional carbohydrate group on DR3-Ser 96 interferes with sDM-enhanced release of prebound peptides from DR3.

Our data indicate a functional role for DM and can account for the phenotypes of B-cell lines carrying DM mutations or a DR3-Ser 96 mutation. Presumably, DM induces conformational changes in DR which result in enhanced dissociation of bound peptides. The low pH optimum for DR/DM interactions is consistent with the colocalization of DR and DM to late endosomal compartments^{2,3,16}. It is also possible that DM could enhance the dissociation of whole invariant chain from DR. DM/DR interactions appear to be of low affinity, as stable DM/DR complexes have been difficult to detect, and sDM must be added to at least a tenfold excess over DR for optimal effects *in vitro*.

The binding of peptides to MHC class II is often characterized by very slow dissociation rates²³. As a result, MHC class II molecules that fill with endogenous peptides early in the biosynthetic pathway (such as CLIP) would not be free to bind exogenous peptides that are encountered later. The presence of DM could resolve this dilemma by catalysing the release of prebound peptides and thereby facilitating ligand exchange. APCs select only a relatively small subset of peptides derived from processed protein antigens for recognition by MHC class-II-restricted T cells (reviewed in ref. 24). In some cases these immunodominant epitopes have been shown to correspond to peptides that can produce SDS-stable MHC class II/peptide complexes with relatively long half-lives in cells²⁵⁻²⁸. DM might skew the population of bound peptides towards these immunodominant epitopes by expediting the release of subdominant epitopes. DM might thus

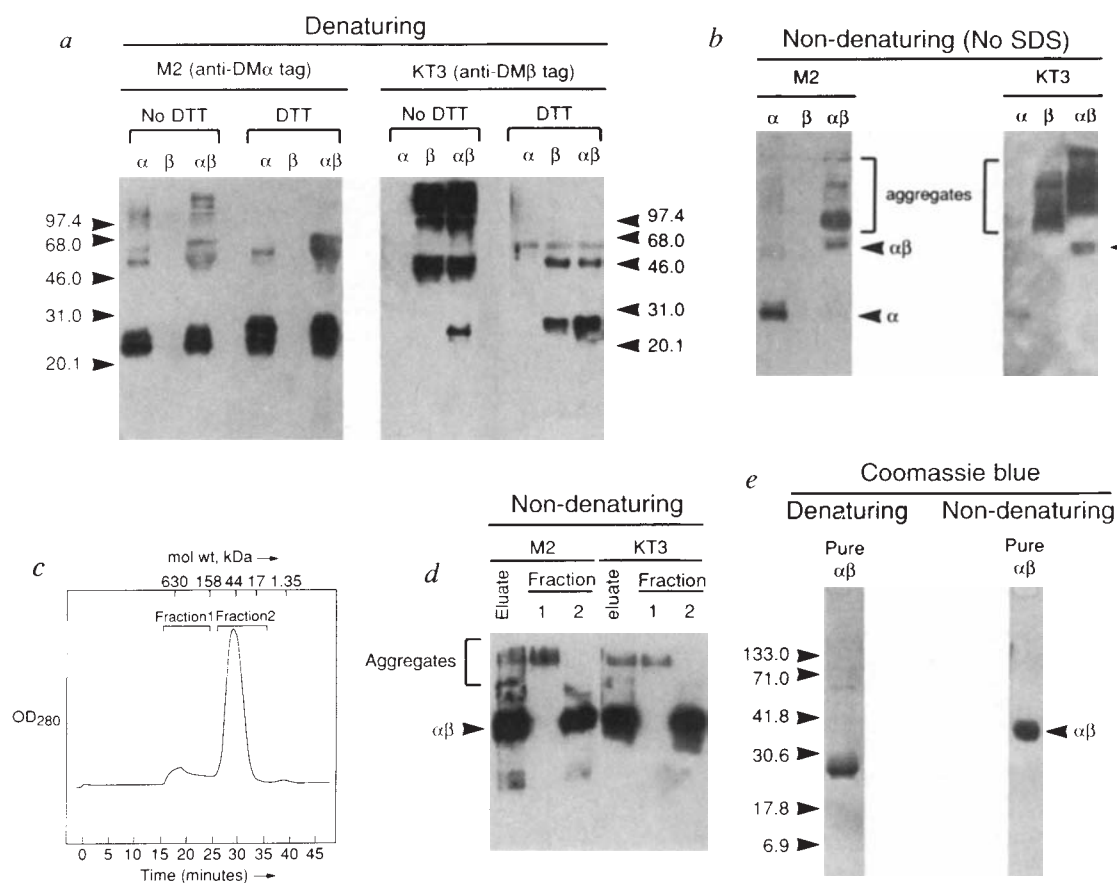


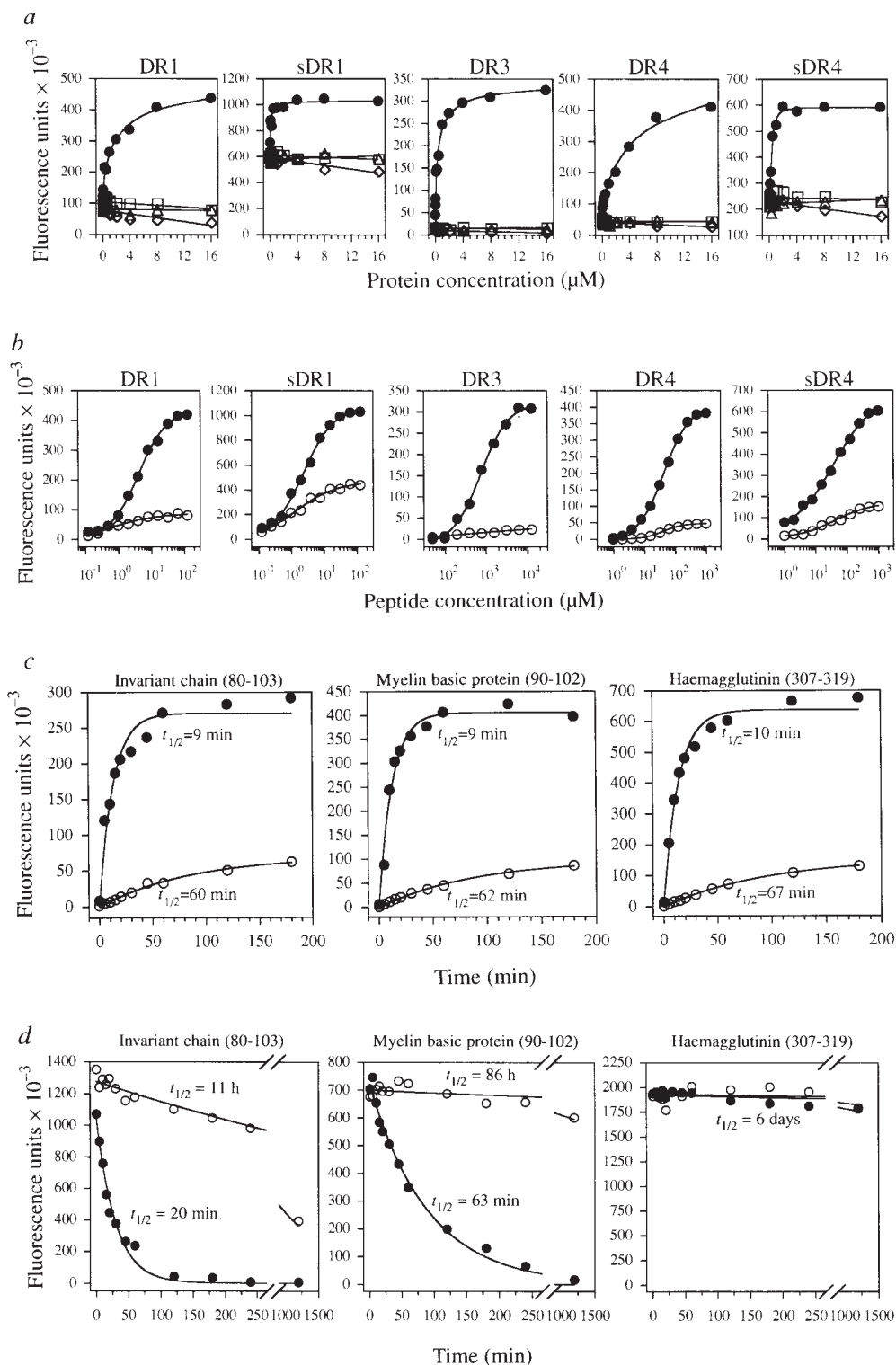
FIG. 1. Expression and purification of recombinant soluble DM. Culture supernatants from S2 cells transfected with plasmids encoding truncated DMA complementary DNA (α), truncated DMB cDNA (β), or cotransfected with both cDNAs ($\alpha\beta$) were applied to SDS denaturing gels (a) or SDS-free non-denaturing gels (b). Protein expression was analysed by immunoblotting with an antibody specific for a peptide tag on the DMA chain (M2) and an antibody specific for a peptide tag on the DMB chain (KT3). DTT (10 mM) was included where indicated. DM α/β heterodimers were purified from supernatants by affinity chromatography using M2 monoclonal antibody columns followed by gel filtration (c). Purified DM was analysed by immunoblotting (d) and visualized by Coomassie blue staining.

METHODS. DMA and DMB cDNA sequences were amplified from Priess cell RNA using reverse transcription polymerase chain reaction (RT-PCR) and cloned into the *EcoRI/BamHI* or *EcoRI/SalI* sites, respectively, of the pRmHA-3 *Drosophila* expression vector²⁹. The DMA-specific PCR primers truncated the cDNA after the 229th codon and incorporated nucleotide sequences encoding the FLAG epitope tag¹⁹ recognized by M2. The DMB-specific PCR primers truncated the cDNA after the 218th codon and included nucleotide sequences encoding the SV40

large T-antigen epitope tag recognized by KT3 (ref. 20). Stable transformants of the S2 *Drosophila* cell line were generated by cotransformation of DM cDNAs along with the pUCHsneo vector³⁰ followed by selection with G418 (Gibco/BRL, Grand Island, New York). Secretion of sDM was induced by addition of 1 mM copper sulphate to the cultures. For denaturing SDS-PAGE, the samples were boiled for 5 min in Laemmli's sample buffer containing 2% SDS (with or without 10 mM DTT) and run on 12% polyacrylamide gels with Tris-glycine SDS running buffer. For non-denaturing PAGE, samples were loaded in sample buffer without SDS and run on 16% polyacrylamide gels with Tris-glycine native running buffer. For western blot analysis, PAGE-separated proteins were transferred to nitrocellulose membranes and probed with either 25 $\mu\text{g ml}^{-1}$ M2 or 10 $\mu\text{g ml}^{-1}$ KT3. Immunoblots were developed using the ECL System (Amersham Life Sciences, Arlington Heights, Illinois). For purification of DM α/β heterodimers, concentrated culture supernatants were applied to PBS-equilibrated M2 affinity columns and then eluted with 100 $\mu\text{g ml}^{-1}$ FLAG peptide. The eluate was applied to a PBS-equilibrated Superdex 300 gel filtration column (Pharmacia, Piscataway, New Jersey) to separate aggregates from heterodimers.

FIG. 2 The effect of sDM on peptide binding to DR. **a**, Binding of biotin-labelled peptides ($1 \mu\text{M}$) to affinity-purified DR molecules (10 nM) in the presence of increasing concentrations of purified sDM (filled circles) and several control proteins (mouse H-2/I-A^d (open diamonds), mouse immunoglobulin (open triangles), bovine serum albumin (open squares)). Binding reactions were incubated for 16 h at 37°C . Biotin-HA (307–319) was used for DR1, sDR1, DR4 and sDR4, and biotin-IgC κ (37–51) for DR3. Assays were developed by incubation with streptavidin-europium followed by measurements of europium fluorescence. All assays were done in duplicate and plotted as mean fluorescence units (FU) minus mean background FU (without DR). Mean background FU were 1,368 for biotin-haemagglutinin (HA) (107–319) and 2,254 for biotin-IgC κ (37–51). Mean FU (without; with $16 \mu\text{M}$ DM) were: DR1 (113,052; 437,799); sDR1 (578,058; 1,029,629); DR3 (12,990; 325,075); DR4 (51,480; 411,636); sDR4 (261,135; 593,638). sDR1 loaded to nearly complete occupancy with biotin-HA (307–319) yielded 1,969,748 FU. **b**, Binding of biotin-labelled peptides to DR as a function of peptide concentration with (filled circles) and without (open circles) $2 \mu\text{M}$ sDM. Binding reactions were incubated for 16 h at 37°C . The peptides used were the same as in **a**. Mean background FU without DR were 1,541 for biotin-HA (103–319) and 1,985 for biotin IgC κ (37–51). The 50% efficiency doses for peptide binding were calculated by fitting the data to a 4-parameter binding equation, and values (without; with $2 \mu\text{M}$ DM) were: DR1/HA (307–319) (3.4 nM; 4.1 nM); sDR1/HA (307–319) (2.3 nM; 2.6 nM); DR3/IgC κ (37–51) (640 nM; 742 nM); DR4/HA (307–319) (45 nM; 40 nM); sDR4/HA (307–319) (39 nM; 42 nM). **c**, Association of biotin-labelled peptides with sDR1 as a function of time. Mean background FU (without DR) were 2,845 for CLIP (80–103); 1,544 for biotin-HA (307–319); and 1,012 for MBP (90–102). Half-times ($t_{1/2}$) were calculated by fitting the data to an equation describing an exponential rise to a maximum. **d**, Dissociation of peptides from sDR1 as a function of time. Mean background FU (without DR) were 1,447 for CLIP (80–103); 1,720 for biotin-HA (307–319); and 989 for MBP (90–102). Half-times were calculated by fitting the data to an exponential decay equation. The control proteins H-2/I-A^d, immunoglobulin, albumin and sDR4 had no effect on peptide dissociation rates (data not shown).

METHODS. Full-length DR molecules were purified from Priess cells (DR4), IBW4 cells (DR1) or 8.1.6 cells (DR3) using LB3.1 affinity columns (DR1, DR4) or L243 affinity columns (DR3) as described previously¹³; sDR1 and sDR4 were affinity purified from supernatants of S2 transformants. Mouse H-2/I-A^d was purified from L10A cells using MKD6 affinity columns. The peptides used were HA (307–319) [PKYVKQNTLKLAT], human IgC κ (37–51) [KVVQWVDNALQSGNS], human CLIP (80–103) [LPKPPKPVSKMRMATP-LLMQALPM], and myelin basic protein (90–102) [HFFKNIVTPRTPA]. For the binding reactions, 10 nM DR was incubated with biotin-labelled peptides at 37°C in 0.15 M citrate-phosphate/ 0.5% NP40/ 0.05% Na N_3 , then neutralized with an equal volume of 50 mM Tris pH 8.0/ 0.05% Na N_3 and immobilized on microtitre plates coated with a $2.5\text{-}\mu\text{g ml}^{-1}$ solution of anti-DR monoclonal antibody (LB3.1). To measure peptide association rates, 200-nM solutions of the indicated biotin-labelled peptides were incubated with



10 nM sDR1 with and without $2 \mu\text{M}$ sDM in binding buffer. At the indicated time points, the reactions were stopped by transferring portions to an LB3.1-coated microtitre plate containing the corresponding unlabelled peptide at $20 \mu\text{M}$. For peptide dissociation rates, sDR1 molecules were incubated with the indicated biotin-labelled peptides overnight at 37°C in binding buffer. Samples were then diluted with excess unlabelled peptide with and without $2 \mu\text{M}$ DM. Final concentrations after dilution were 10 nM DR1, 100 nM biotin peptide, $100 \mu\text{M}$ unlabelled peptide. At the indicated time points, the reactions were stopped by transferring portions to an LB3.1-coated microtitre plate at 4°C . All plates were developed by incubation for 1 h at 4°C with $0.1 \mu\text{g ml}^{-1}$ streptavidin-europium (Wallac, Gaithersburg, Maryland) in PBS with $7 \mu\text{g ml}^{-1}$ DTPA (Sigma, St Louis, Missouri)/ 0.1% BSA/ 0.01% Na N_3 followed by addition of enhancement buffer (Wallac). Time-resolved fluorescence was quantified using a DELFIA Research Fluorometer.

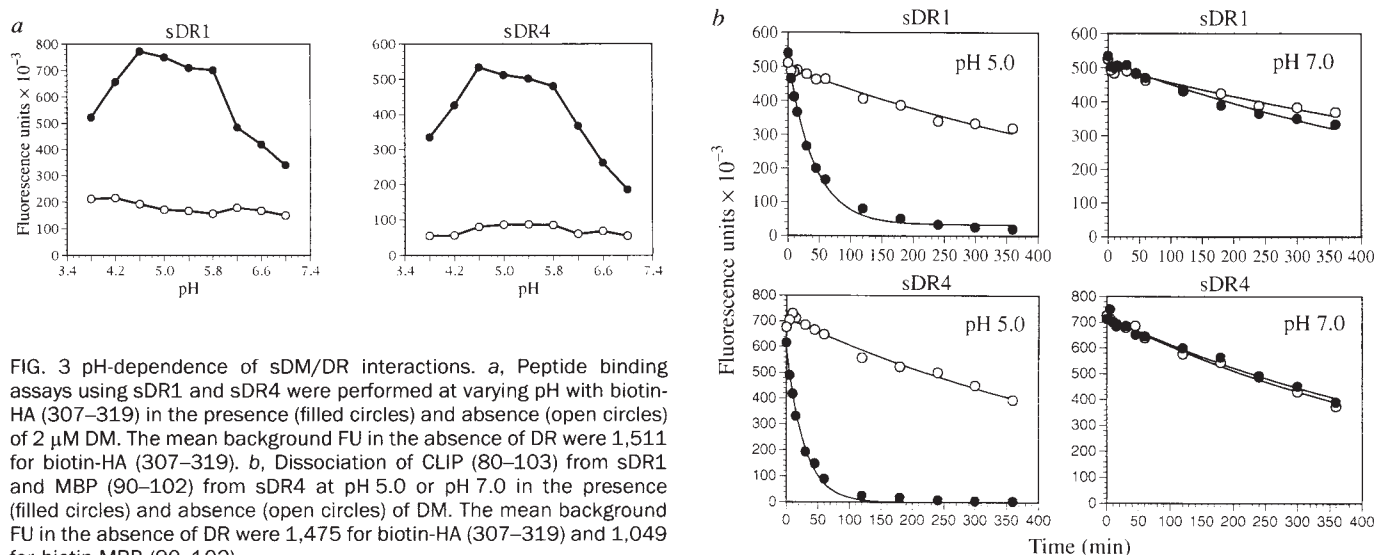


FIG. 3 pH-dependence of sDM/DR interactions. **a**, Peptide binding assays using sDR1 and sDR4 were performed at varying pH with biotin-HA (307–319) in the presence (filled circles) and absence (open circles) of 2 μ M DM. The mean background FU in the absence of DR were 1,511 for biotin-HA (307–319). **b**, Dissociation of CLIP (80–103) from sDR1 and MBP (90–102) from sDR4 at pH 5.0 or pH 7.0 in the presence (filled circles) and absence (open circles) of DM. The mean background FU in the absence of DR were 1,475 for biotin-HA (307–319) and 1,049 for biotin-MBP (90–102).

METHODS. For peptide binding assays, sDR1 or sDR4 was incubated with 1 μ M biotin-HA (307–319) for 4 h at 37 °C in 0.15 M citrate-phosphate binding buffers of various pHs. After neutralization with an equal volume of 50 mM Tris pH 8.0/0.05% Na₂S₂O₅, the assays were developed as described above. For peptide dissociation assays, sDR1 (100 nM) was incubated with biotin-CLIP (80–103) (200 nM) and sDR4 was incubated with biotin-MBP (90–102) (200 nM) for 16 h at 37 °C. The samples were divided in half, and the buffer was exchanged to 0.15 M citrate-phosphate buffer/0.05% Na₂S₂O₅ at either pH 5.0 or pH 7.0 using

Biospin 30 columns (Biorad, Hercules, California). Samples were then diluted to a final concentration of 10 nM DR with pH 5.0 or pH 7.0 buffered solutions containing the corresponding unlabelled peptide (10 μ M final concentration) with or without sDM (2 μ M final concentration). At the indicated time points, the reactions were stopped by transferring portions to an LB3.1-coated microtitre plate at 4 °C. The assays were developed as described above.

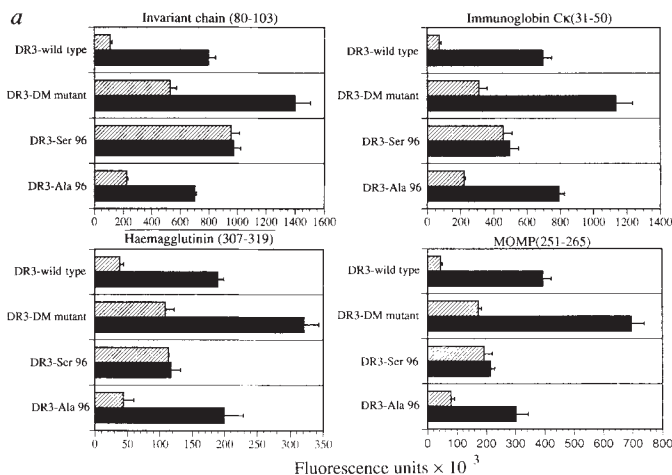
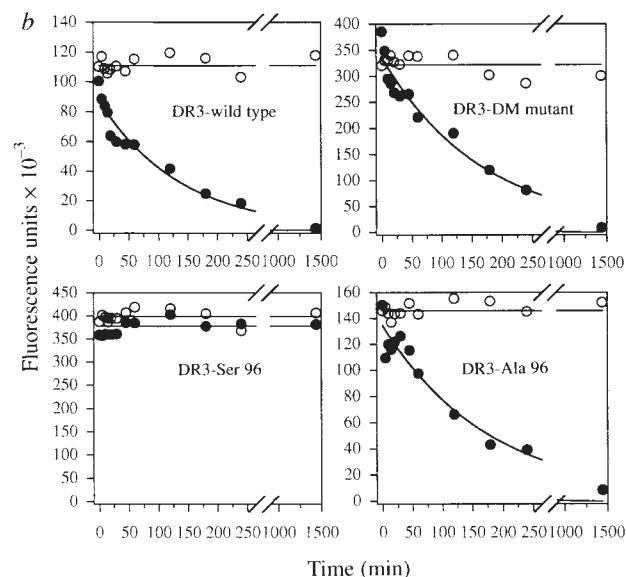


FIG. 4 sDM does not interact with DR3-Ser 96. **a**, Failure of DM to enhance peptide binding to DR3-Ser 96. DR3/peptide binding assays were performed using 10 nM of various forms of DR3 and 10 μ M of the indicated biotin-labelled peptides. DM was added (black) or not (hatched) where indicated. Binding reactions were incubated for 4 h at 37 °C. Mean background FU in the absence of DR3 were 1,269 for biotin-CLIP (80–103), 2,738 for biotin-IgCκ (37–51), 1,436 for biotin-HA (307–310), and 2,617 for biotin-MOMP (251–265). **b**, Failure of DM to accelerate the dissociation of CLIP (80–103) from DR3-Ser 96. Dissociation of biotin-CLIP (80–103) from the various forms of DR3 was measured in the presence (filled circles) and absence (open circles) of 2 μ M DM. Mean background fluorescent units in the absence of DR3 were 1,452 for biotin-CLIP (80–103).

METHODS. DR3 wild type, DR3-DM mutant, DR3-Ser 96, and DR3-Ala 96 were purified using L243 affinity columns from the 8.1.6



(ref. 22), 9.5.3 (ref. 22), 10.24.6 (ref. 22) and 98.99.25 (C. Bergman, T. Saweda, M.-d. Y. Chang and E. Mellins, submitted) cell lines, respectively. Binding assays were performed at 37 °C in binding buffer at pH 5.0 as previously described. Error bars represent the standard deviation of duplicate samples. The MOMP (251–265) peptide sequence is derived from the major outer membrane protein of *Chlamydia trachomatis* [QASLALSYRLNMFPT]. For CLIP dissociation assays, the various DR3 molecules were incubated with the indicated biotin-labelled peptides for 16 h at 37 °C in binding buffer. Samples were then diluted with the corresponding unlabelled peptide with or without DM. Final concentrations after dilution were 10 nM DR3, 100 nM biotin peptide, 100 μ M unlabelled peptide, with or without 2 μ M DM. At the indicated time points, the reactions were stopped by transferring portions to an LB3.1-coated microtitre plate at 4 °C. The assays were developed as described above.

function as a peptide 'editor', ensuring that most MHC class II molecules reach the cell surface loaded with peptides that have very slow dissociation rates. □

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- Kelly, A. P., Monaco, J. J., Cho, S. G. & Trowsdale, J. *Nature* **353**, 571–573 (1991).
- Denzin, L. K., Robbins, N. F., Carboy-Newcomb, C. & Cresswell, P. *Immunity* **1**, 595–606 (1994).
- Karlsson, L., Peleraux, A., Lindstedt, R., Liljedahl, M. & Peterson, P. A. *Science* **266**, 1569–1573 (1994).
- Riberdy, J. M., Newcomb, J. R., Surman, M. J., Barbosa, J. A. & Cresswell, P. *Nature* **360**, 474–477 (1992).
- Mellins, E., Kempin, S., Smith, L., Monji, T. & Pious, D. J. *exp. Med.* **174**, 1607–1615 (1991).
- Ceman, R. D., Rudersdorf, R., Long, E. O. & Demars, R. J. *Immun.* **149**, 754–761 (1992).
- Riberdy, J. M. & Cresswell, P. J. *Immun.* **148**, 2586–2590 (1992).
- Mellins, E. et al. *Nature* **343**, 71–74 (1990).
- Sette, A. et al. *Science* **259**, 1801–1804 (1992).
- Monji, T., McCormack, A. L., Yates, J. R. I. & Pious, D. J. *Immun.* **153**, 4468–4477 (1994).
- Morris, P. et al. *Nature* **368**, 551–554 (1994).
- Fling, S. P., Arp, B. & Pious, D. *Nature* **368**, 554–558 (1994).
- Amigorena, S., Drake, J. R., Webster, P. & Mellman, I. *Nature* **369**, 113–120 (1994).

- Peters, P. J., Neefjes, J. J., Oorschot, V., Ploegh, H. L. & Geuze, H. J. *Nature* **349**, 669–676 (1991).
- Qui, Y., Yu, X., Wandinger-Ness, A., Dalke, D. P. & Pierce, S. K. *J. Cell Biol.* **125**, 595–605 (1994).
- Sanderson, F. et al. *Science* **266**, 1566–1573 (1994).
- Tulp, A., Verwoerd, D., Dobberstein, B., Ploegh, H. L. & Pieters, J. *Nature* **369**, 120–126 (1994).
- West, M. A., Lucocq, J. M. & Watts, C. *Nature* **369**, 147–151 (1994).
- Hopp, T. P. et al. *Biotechnology* **6**, 1205–1210 (1988).
- MacArthur, H. & Walter, G. J. *Virology* **52**, 483–491 (1984).
- Stern, L. J. & Wiley, D. C. *Cell* **68**, 465–477 (1992).
- Mellins, E. et al. *J. exp. Med.* **179**, 541–549 (1994).
- Buus, S., Sette, A., Colon, S. M., Jenis, D. M. & Grey, H. M. *Cell* **47**, 1071–1077 (1986).
- Sercarz, E. E. et al. *A. Rev. Immun.* **11**, 729–766 (1993).
- Nelson, C. A., Petzold, S. J. & Unanue, E. R. *Proc. natn. Acad. Sci. U.S.A.* **90**, 1227–1231 (1993).
- Nelson, C. A., Petzold, S. J. & Unanue, E. R. *Nature* **371**, 250–252 (1994).
- Germain, R. N. & Rinker, A. G. J. *Nature* **363**, 725–728 (1993).
- Lanzavecchia, A., Reid, P. A. & Watts, C. *Nature* **357**, 249–252 (1992).
- Bunch, T. A., Grinblat, Y. & Goldstein, L. S. B. *Nucleic Acids Res.* **16**, 1043–1061 (1988).
- Steller, H. & Pirrotta, V. *EMBO J.* **4**, 167–171 (1985).

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A SNARE-like protein required for traffic through the Golgi complex

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THE secretory pathway of eukaryotic cells comprises several distinct membrane-bound compartments which are interconnected by transport vesicles that pinch off from one membrane and fuse with the next. Targeting of these vesicles is mediated in part by interactions between integral membrane proteins on the vesicles and target organelles (soluble NSF attachment protein receptors (SNAREs)), termed v-SNAREs and t-SNAREs, respectively^{1–4}. SNAREs required for endoplasmic reticulum (ER)–Golgi transport and for fusion of vesicles with the plasma membrane are already known. Here we identify two yeast membrane proteins that show genetic interactions with Sed5p, which is the t-SNARE for ER–Golgi traffic^{3,4}. One of these membrane proteins, Sft1p, is structurally similar to the known v-SNAREs and is required for transport from an early to a later Golgi compartment. Our results indicate that a single t-SNARE can control more than one transport step, and provide the first candidate for a SNARE involved in intra-Golgi traffic.

Temperature-sensitive (ts) alleles of *SED5* were created by polymerase chain reaction (PCR)-mediated random mutagenesis⁵, and one of these, *sed5-1*, could be ascribed to a single R→G change at position 255. Two multicopy suppressors of this mutation were isolated and named *SFT1* and *SFT2* (suppressor of *sed5* ts; see Fig. 1 legend for details). Overexpression of *SFT2* also efficiently suppressed the growth phenotype of a second *sed5* ts mutant (*sed5-5*), but *SFT1* did not (Fig. 1b), indicating that the genetic interaction between Sed5p and Sft1p is allele specific. Neither *SFT1* nor *SFT2* could suppress a complete deletion of *SED5*.

Sft2p is a protein of relative molecular mass 24,000 (M_r 24K) which has four predicted transmembrane domains, and is encoded by open reading frame YBL102w on chromosome II; further studies of this protein will be reported elsewhere. *SFT1* encodes a protein (M_r 11K) (Fig. 1a) which consists largely

a

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      . . . . .
MSNSRYSTESNNDRKLEGLANKLATFRNINQEIG 35
      . . . . .
      . . . . .
DRAVSDSSVINQMTDSLGMFTDIKNSSRLTRSL 70
      . . . . .
      . . . . .
KAGNSIWRMVGALLLFFILYTLFKLF 97
      . . . . .

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b

	<i>sed5-1</i>	<i>sed5-5</i>	<i>sft1-1</i>	<i>sft1-2</i>
<i>SFT1</i>	++	–	++	++
<i>SFT2</i>	+	++	–	–
<i>SED5</i>	++	++	+	–

FIG. 1 Sft1p and its genetic interactions. a, The sequence of Sft1p. The predicted transmembrane domain is underlined, and the first and fourth positions of the heptad repeats are indicated by dots. The open reading frame for Sft1p corresponds to residues 430,273 to 430,237 and 430,096 to 429,838 of chromosome XI. The intron lies between the Asn residues at positions 12 and 13. Although the gene lies in a region of the genome that has been sequenced (between YKLO06w and CAP1), the corresponding open reading frame has not previously been described. b, Growth at 37 °C on rich plates of the indicated mutants carrying multicopy plasmids with the *SFT1*, *SFT2* or *SED5* genes. ++ indicates good growth, + poor growth, and – no detectable growth. None of the mutants grew under these conditions in the absence of a suppressing gene.

METHODS. The *sed5-1* mutation was introduced into the chromosomal *SED5* gene in strain SEY6210 (ref. 3) by the pop-in/pop-out method¹⁵, and the resultant ts strain was transformed with a library of yeast genomic DNA on a multicopy plasmid. Plasmids were isolated from colonies that grew at the non-permissive temperature (37 °C). The suppressor activity was localized by subcloning, and in the case of *SFT1* confirmed by assaying the activity of a PCR-generated copy of the reading frame under the control of the triose phosphate isomerase (TPI) promoter. The position of the intron was confirmed by PCR amplification of the reading frame from a complementary-DNA library, followed by sequencing.

of a predicted amphipathic coiled-coil structure followed by a carboxy-terminal membrane anchor, a structure similar to that of known v-SNAREs⁶. Sft1p is not related to the syntaxin family of t-SNAREs, of which Sed5p is a member^{7,8}.

The predicted transmembrane domain of Sft1p is short and rich in phenylalanine, features that are characteristic of Golgi membrane proteins⁹. To see whether Sft1p is a Golgi resident,

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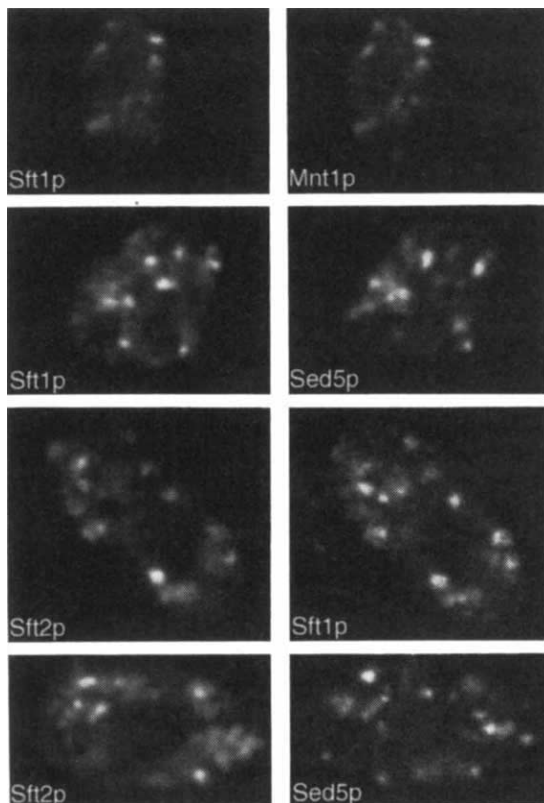
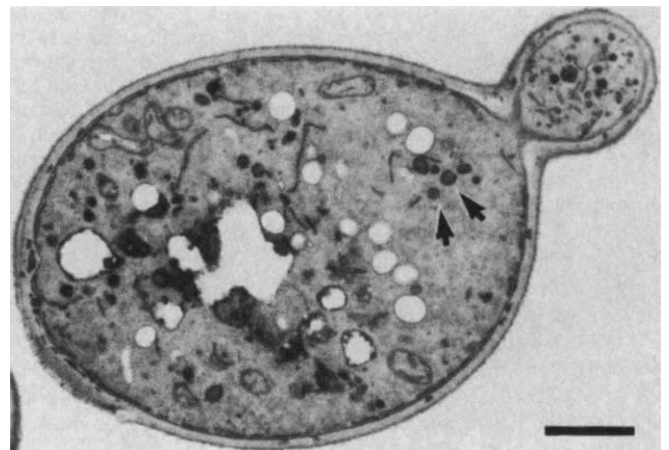


FIG. 2 Immunofluorescence analysis of Sft1p and Sft2p in yeast cells. Left and right images correspond to double labelling of the indicated proteins, visualized by confocal microscopy³. In the top four panels, myc-tagged Mnt1p³ and Sed5p¹⁰ (expressed from the TPI promoter on a multicopy vector, or, in the case of Mnt1p, integrated into the genome) were detected with the monoclonal antibody 9E10, and Sft1p (expressed from its own promoter on a multicopy vector) was detected with affinity-purified rabbit antibodies raised against a bacterially expressed fusion protein comprising the entire sequence of Sft1p fused at its N terminus to glutathione S-transferase (GST). In the bottom four panels myc-tagged Sft2p (expressed from the TPI promoter on a multicopy vector) was detected with mAb 9E10, and untagged Sft1p and Sed5p (expressed from their own promoters on multicopy plasmids) were detected with the appropriate rabbit antibodies³. Only a few cells expressed high levels of both Sft1p and Sed5p, for unknown reasons. Note that apparent colocalization or partial colocalization does not necessarily imply that two proteins are in the same compartment, because individual Golgi elements may not be optically resolved. Full width of each panel corresponds to 12 μ m.

FIG. 3 An electron micrograph of an *sft1-2* cell incubated at 37 °C. Note the normal appearance of the ER, and the accumulation of vesicular and larger circular structures which stain darkly with the permanganate fixation method¹⁶ (arrows). Cells with this appearance were common 2 h after temperature shift; at later time points there was a progressive accumulation of the circular structures. The *sft1-2* gene was expressed from a centromere-containing vector in a derivative of strain SEY6210³ in which the entire chromosomal *SFT1* open reading frame had been replaced by the *LEU2* gene. Scale bar, 1 μ m.



we prepared antibodies against it and examined yeast cells by immunofluorescence. The endogenous protein was present at too low a level to be detected, but cells expressing the gene from a multicopy plasmid showed a punctate staining pattern characteristic of the Golgi complex. In cells that also expressed an epitope-tagged version of the Golgi enzyme Mnt1p, clear colocalization of Mnt1p and Sft1p was observed (Fig. 2). This distribution is different from that of Sed5p, which colocalizes poorly with Mnt1p³, and indeed double staining of Sft1p and Sed5p showed only partial overlap (Fig. 2). A further marker was provided by an epitope-tagged version of Sft2p: there was great similarity in the distribution of Sft2p and Sft1p, but only partial correspondence between Sft2p and Sed5p (Fig. 2). These results suggest that both Sft1p and Sft2p may reside in a later Golgi compartment than the one occupied by Sed5p.

Disruption of the *SFT1* gene indicated that it was essential for normal growth. To allow its function to be studied, we prepared two temperature-sensitive alleles (*sft1-1* and *sft1-2*) by

PCR mutagenesis⁵. Strikingly, we found that the growth defect of *sft1-1* could be alleviated by overexpression of *SED5* (Fig. 1b), emphasizing the reciprocal nature of the interactions between these two genes.

We then studied the phenotypes of the *sft1* mutants at the non-permissive temperature. By electron microscopy the ER appeared normal, but there was a marked accumulation of circular or horseshoe-shaped membrane structures 120–180 nm in diameter, together with some short linear membrane profiles and smaller vesicles (Fig. 3). The circular structures are similar to those seen when Sed5p is overexpressed (ref. 3 and data not shown), and may represent aberrant forms of Golgi membrane (or, by analogy with animal cells¹⁰, the ER–Golgi intermediate compartment). The appearance of the *sft1* mutant cells contrasts with that of cells depleted of Sed5p, which show a massive accumulation of ER and vesicles, but not of the putative Golgi structures³. Thus the normal function of Sft1p is different from that of Sed5p.

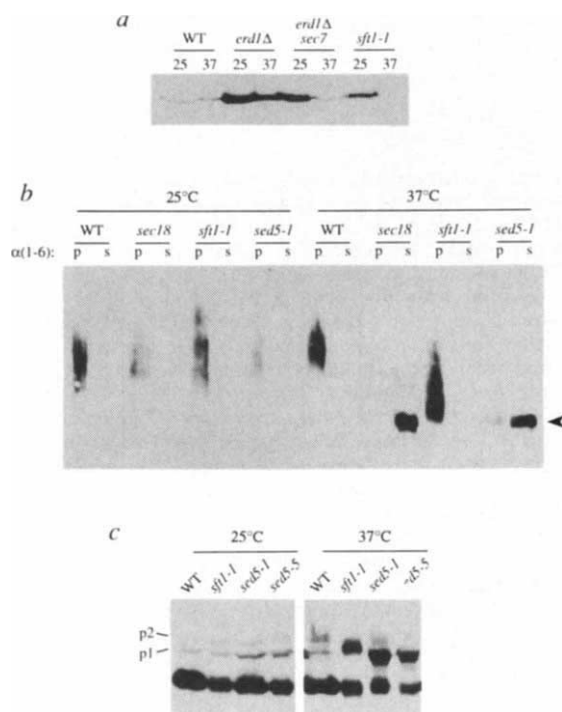
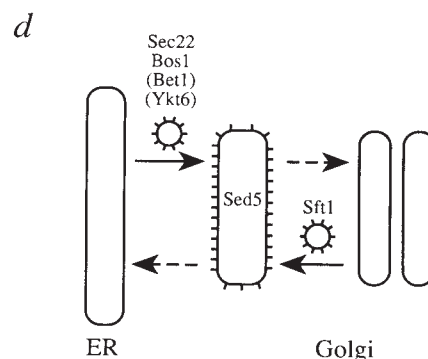


FIG. 4 Secretion phenotype of *sft1-1*. **a**, Cells of the indicated strains were grown at 25 °C or shifted to 37 °C for 1 h, then centrifuged, resuspended in fresh medium and incubated at the same temperatures for a further 2 h. The cells were then removed by centrifugation and the growth medium subjected to immunoblot analysis with an antibody specific for yeast BiP (Kar2p)¹⁷. Wild-type cells (WT) secrete little BiP, whereas *erd1Δ* cells secrete much more¹⁶; this secretion is blocked by the *sec7* mutation. A moderate amount of BiP is secreted by *sft1-1* cells at 25 °C, but not at the non-permissive temperature. **b**, Effects on invertase processing. The indicated mutants were transformed with a plasmid expressing the secreted form of invertase tagged with a c-myc epitope (a gift from R. Chapman). They were grown at 25 °C, or shifted to 37 °C for 2 h. Total cellular extracts were prepared, immunoprecipitated

At the permissive temperature *sft1-1* cells secreted the ER protein BiP, as do several secretory mutants¹¹. BiP secretion was reduced by more than 95% at 37 °C, however, indicating that Sft1p function is required for secretion (Fig. 4a). Transport of an epitope-tagged secreted form of invertase in *sft1-1* cells was then examined; for comparison, *sec18* and *sed5-1* strains were analysed in parallel (Fig. 4b). Samples were precipitated with antibodies specific for $\alpha(1\rightarrow6)$ -linked mannose residues, which are added only in post-ER compartments. At low temperature all strains contained mature invertase, which is heterogeneous in size owing to the variable-length outer carbohydrate chains added in the Golgi. At 37 °C, the *sec18* strain accumulated the core-glycosylated ER form of the protein, lacking $\alpha(1\rightarrow6)$ Man, as expected because this mutant is defective in transport to the Golgi complex¹². The *sed5-1* strain showed a similar phenotype, except that some $\alpha(1\rightarrow6)$ Man-containing material very slightly larger than the ER form was also observed (Fig. 4b). About 15% of the accumulated invertase was in this form after 2 h at 37 °C; after 4 h incubation the proportion increased to 40% (data not shown). This material presumably represents invertase molecules that have reached the early Golgi compartment where the first $\alpha(1\rightarrow6)$ Man residue is added^{13,14}, but have failed to move on. The accumulation of such molecules implies that Sed5p has a role not just in ER-Golgi transport, but also in a subsequent step. Overexpression of *SFT1* in the *sed5-1* strain pre-



tated with antibodies specific for $\alpha(1\rightarrow6)$ mannose¹⁸, and the precipitates (p) and supernatants (s) were analysed by immunoblotting with the anti-myc mAb 9E10. At the permissive temperature, all strains yielded mature, $\alpha(1\rightarrow6)$ Man-containing invertase, which is heterogeneous in size. At 37 °C, the *sec18* and *sed5-1* strains accumulate significant amounts of core-glycosylated enzyme (*M*, 80K, arrow). **c**, Effects on CPY processing. The indicated mutants were incubated at 25 °C, or for 3 h at 37 °C, and total cell extracts were analysed by immunoblotting with anti-CPY antibody³. CPY is proteolytically processed in the vacuole, and the most prominent band represents this mature enzyme. The p1 (ER) and p2 (Golgi) precursors are indicated. Results similar to those shown for *sft1-1* were also obtained with the *sft1-2* allele. **d**, Model for Sft1p function. Sed5p acts as the t-SNARE for an early Golgi compartment (equivalent to the 'intermediate compartment' of animal cells¹⁰), which probably corresponds to the site where the first $\alpha(1\rightarrow6)$ Man residue is added to core oligosaccharides. Vesicles from the ER are targeted to this compartment by some or all of the v-SNAREs indicated, which interact with Sed5p⁴. Note that the presence of Bet1p on ER-derived vesicles may depend on the manner in which those vesicles are formed^{19,20}, and that Ykt6p has not been shown to be on these vesicles, although it is likely to be involved in this step because its overexpression can suppress the growth defect of *sec22-1* (D.K.B., unpublished observations). Sft1p is postulated to allow retrograde transport vesicles from a later Golgi compartment to fuse with the Sed5p-containing membrane, by acting as a v-SNARE that specifically recognizes Sed5p. This step is assumed to be necessary to recycle components needed for forward transport, thus explaining the requirement for both Sed5p and Sft1p for transport out of the Sed5p compartment. In *sed5-1*, Sft1p cycling through this compartment would interact with and stabilize the ts form of Sed5p, thus helping it to perform all of its functions.

vented the accumulation of both core-glycosylated invertase and this intermediate form at 37 °C (data not shown).

In contrast to the *sed5* phenotype, the *sft1-1* strain accumulated only $\alpha(1\rightarrow6)$ Man-containing forms of invertase at 37 °C (Fig. 4b). These were smaller than the mature enzyme, and included molecules of similar size to the ER form, indicating that the Golgi modifications were incomplete. They were not secreted, but accumulated inside spheroplasts (data not shown). A similar result was obtained when the vacuolar enzyme carboxypeptidase Y (CPY) was analysed: the *sft1-1* cells accumulated a form of this protein that was larger than the p1 ER-glycosylated precursor, but smaller than the p2 precursor that is found in a late Golgi compartment¹³ (Fig. 4c). Endoglycosidase H treatment showed that carbohydrate modifications were responsible for the unusual mobility of this protein (data not shown); it presumably corresponds to a normally short-lived intermediate that is trapped in an early Golgi compartment in the mutant. As expected, the *sed5-1* and *sed5-5* strains accumulated predominantly the p1 form of CPY (the mature CPY visible in Fig. 4c represents material accumulated before the temperature shift which is also detected by the immunoblot assay). Taken together, these results indicate strongly that Sft1p is required for the transport of proteins between an early and a later Golgi compartment.

The allele-specific and reciprocal nature of their genetic interactions suggests that Sft1p comes into direct contact with Sed5p. It has been reported that immunoprecipitation of Sed5p from extracts of *sec18* cells results in the coprecipitation of several polypeptides, including the known ER-derived v-SNAREs, and two unknown proteins termed p14 and p28 whose sizes are similar to those of Sft1p and Sft2p⁴. Peptide sequences subsequently determined for the latter proteins indicate that p28 is not Sft2p; however, p14 is identical to Sft1p (M. Sogaard, J. Rothman and T. Söllner, personal communication). This confirms that Sft1p binds to Sed5p; moreover, this binding is sensitive to Sec18p⁴, a homologue of the *N*-ethylmaleimide (NEM)-sensitive fusion protein (NSF). Sft1p therefore has both the biochemical and functional properties expected of a SNARE^{1,2}.

The phenotype and genetic interactions of *sed5-1* indicate that Sed5p is involved not just in ER-Golgi transport, but also in the intra-Golgi step that requires Sft1p. One possible explanation for this is that Sed5p acts as a t-SNARE both for ER-derived vesicles and for retrograde vesicles coming from a later Golgi compartment, one of whose functions would be to recycle components involved in forward traffic. The location and properties of Sft1p are consistent with it being a v-SNARE for such retrograde vesicles (Fig. 4d). Alternatively, it might cooperate

with Sed5p as a t-SNARE specific for this step. In either case, by binding to and stabilizing the ts form of Sed5p, it could correct all the defects of *sed5-1*. □

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1. Rothman, J. E. *Nature* **372**, 55–63 (1994).
2. Söllner, T. et al. *Nature* **362**, 318–324 (1993).
3. Hardwick, K. G. & Pelham, H. R. B. *J. Cell Biol.* **119**, 513–521 (1992).
4. Sogaard, M. et al. *Cell* **78**, 937–948 (1994).
5. Muhirad, D., Hunter, R. & Parker, R. *Yeast* **8**, 79–82 (1992).
6. Dascher, C., Ossig, R., Gallwitz, D. & Schmitt, H. D. *Molec. cell. Biol.* **11**, 872–885 (1991).
7. Pelham, H. R. B. *Cell* **73**, 425–426 (1993).
8. Bennett, M. K. et al. *Cell* **74**, 863–873 (1993).
9. Bretscher, M. S. & Munro, S. *Science* **261**, 1280–1281 (1993).
10. Banfield, D. K., Lewis, M. J., Rabouille, C., Warren, G. & Pelham, H. R. B. *J. Cell Biol.* **127**, 357–371 (1994).
11. Semenza, J. C., Hardwick, K. G., Dean, N. & Pelham, H. R. B. *Cell* **61**, 1349–1357 (1990).
12. Novick, P., Ferro, S. & Schekman, R. *Cell* **25**, 461–469 (1981).
13. Graham, T. R. & Emr, S. D. *J. Cell Biol.* **114**, 207–218 (1991).
14. Franzusoff, A. & Schekman, R. *EMBO J.* **8**, 2695–2702 (1989).
15. Rothstein, R. *Meth. Enzym.* **194**, 281–301 (1991).
16. Sweet, D. J. & Pelham, H. R. B. *EMBO J.* **11**, 423–432 (1992).
17. Hardwick, K. G., Lewis, M. J., Semenza, J., Dean, N. & Pelham, H. R. B. *EMBO J.* **9**, 623–630 (1990).
18. Dean, N. & Pelham, H. R. B. *J. Cell Biol.* **111**, 369–377 (1990).
19. Lian, J. P. & Ferro-Novick, S. *Cell* **73**, 735–745 (1993).
20. Barlowe, C. et al. *Cell* **77**, 895–907 (1994).

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Powering the flagellar motor of *Escherichia coli* with an external voltage source

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ROTARY motors of bacterial flagella are driven by ions that move across the cytoplasmic membrane down an electrochemical gradient^{1–4}. For *Escherichia coli*, the ions are protons, and the maximum work per unit charge that they can do is the proton-motive force. To test whether motor efficiency is limited by proton leakage or mechanical nonlinearities, we measured torque as a function of proton-motive force. Filamentous cells were drawn into micropipettes and energized with an external voltage source. Torque was proportional to proton-motive force up to -150 mV, twice the span accessible by earlier techniques^{5–9}. This is consistent with a mechanism in which a fixed number of protons, working at unit efficiency, carry the motor through each revolution. We also found that individual torque-generating elements inactivate at low potentials or potentials of reverse sign. When normal potentials are restored, they reactivate sequentially.

Filamentous cells were pulled part of the way into glass micropipettes that had constrictions near their tips which closely matched the cell diameter (Fig. 1a). The constriction held a cell in place and divided it into an outer and an inner segment (Fig. 1b). The outer segment carried a marker tethered to a motor, by which motor rotation was visualized (Fig. 1c). The inner segment was exposed to the ionophore gramicidin S, a cyclic peptide known to permeabilize both the outer and cytoplasmic membranes^{10,11}. An external command voltage was applied between an electrode in the micropipette and an electrode in the external bath (Fig. 1d).

The resistance of the micropipette alone was about 50 M Ω . With a cell in place, the initial total resistance was about 280 M Ω . The access resistance of the micropipette (estimated to be <5 m Ω) was much smaller than this, so the voltage across

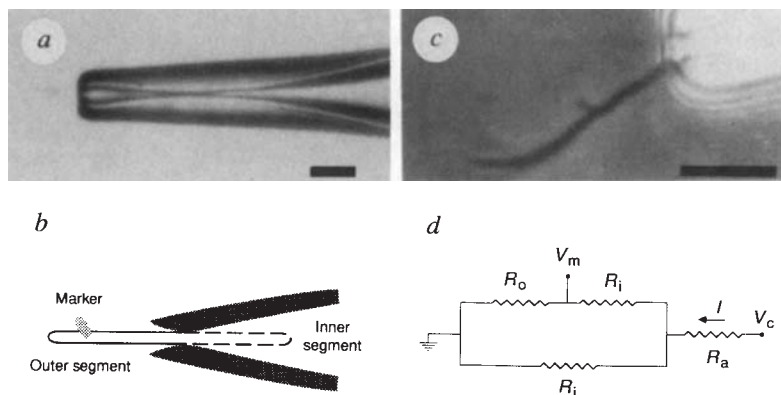
the cell was well approximated by the command voltage. The resistances of the inner and the outer segments formed a voltage divider. Making the inner segment permeable did not change the total resistance by more than a few megohms. Thus the outer segment was not affected initially by gramicidin S and essentially all of the command voltage fell across it. Because the cytoplasm of the cell is a much better conductor than the non-permeabilized outer-segment membrane, most of the command voltage appeared across the motor¹². After the experiment was completed, when the cell was fully permeabilized, the total resistance fell to a value only a few megohms higher than that of the micropipette.

Time was required to select and then pull a filamentous cell into the micropipette, during which fluid from the external medium was drawn into its tip. As a result, it took a few minutes for gramicidin S to reach the cell and for motor rotation to cease, owing to the collapse of both potential and pH components of the endogenous proton-motive force. Once the motors stopped, they could be restarted by application of a negative command voltage. To our surprise, however, there was a significant time lag between the application of a negative command voltage and the beginning of rotation, and so we tried to understand this phenomenon. The distribution of lag times is shown in Fig. 2a. The number of motors that remained stopped decayed exponentially with a time constant of 8 s, although a faster process occurred in the first 2 s. Subsequently, the motor increased its speed in discrete steps over the course of tens of seconds, as shown by the example in Fig. 2b. These steps were sudden and occurred within the time resolution of the video (33 ms). In most cases the steps were of equal size, indicating that torque was added in equal increments (Fig. 2c). Stopped motors could not be made to rotate with positive command voltages. However, if motors were first energized with a negative command voltage and then the polarity was reversed, motor reversal was sometimes observed: in 5 of 17 trials the motors reversed direction, but only for a few seconds before coming to a stop; in 3 cases the markers continued in their original direction for 1, 2 or 6 video frames, possibly as a result of relaxation of their tethers^{13,14}; in the remaining cases the motors stopped immediately.

These results show that torque-generating elements in the motor are inactivated when the cell's proton-motive force is dissipated or of positive sign. These elements are reactivated by a negative proton-motive force. The elements in question are likely

FIG. 1 a, Photomicrograph of a micropipette tip; scale bar, 10 μm . b, Schematic diagram of the micropipette with partially inserted cell. c, Video print of a cell with an attached marker; scale bar, 10 μm . d, Schematic diagram of the equivalent electrical circuit. R_a is the access resistance of the micropipette, R_i and R_o are the resistances of the inner and outer segments of the cell, respectively, and R_l is the leakage resistance. V_c is the command voltage, and V_m is the voltage across the motor. The total resistance R_T was calculated from V_c and total current I measured by the patch clamp.

METHODS. Cells of *E. coli* strain HCB892, a polyhook (*fliK*) strain deleted for the F_0F_1 H^+ -ATPase (*uncB-uncC*)¹², were grown at 30 °C in Luria broth (1% tryptone, Difco; 0.5% yeast extract, Difco; 0.5% NaCl) supplemented with 0.2% fructose until early exponential phase. Cephalixin (50 $\mu\text{g ml}^{-1}$, Sigma), a β -lactam antibiotic that suppresses septation, was added, and incubation was continued for about 4 h until the cells had reached a length of 70–90 μm . The cells were washed twice by centrifugation (2,000 g, 8 °C, 10 min) and resuspended in tethering buffer (10 mM potassium phosphate, pH 7.0, 67 mM NaCl, 0.1 mM EDTA) at a density of 10^6 cells cm^{-3} . Markers were prepared from polyhook strain HCB9 (*fliC fliK*)²⁶ grown in tryptone broth (1% tryptone, 0.5% NaCl) at 30 °C until late exponential phase, treated with 0.1% glutaraldehyde (Sigma grade I) for 30 min, washed by filtration²⁷ and resuspended in tethering buffer at a density of $2 \times 10^5 \text{ cm}^{-3}$. Portions (200 μl) of cell and marker suspensions were combined with 30 μl of a 1:100 dilution of anti-hook antibody²⁶ and the preparation was allowed to stand for 1 h. The markers in this preparation spun predominantly counterclockwise. Micropipettes were pulled from borosilicate glass tubing (1 mm diameter; Kimax-51), and their tips were squared off at a diameter of about 10 μm ²⁸. The tips were then heated on two sides by V-shaped heating filaments (Pt wire, 0.05 mm diameter) to produce a narrow constriction slightly larger than the diameter of a cell near the end of the micropipette. Micropipettes were



filled with tethering buffer containing 8–15 $\mu\text{g ml}^{-1}$ gramicidin S (Sigma) and coated near their tips with beeswax. Filamentous cells with spinning markers, suspended in an open dish (16 mm internal diameter $\times$ 2 mm high), were pulled part of the way into micropipettes using transient gentle suction. Experiments were performed at room temperature (22 °C) under an inverted microscope (Nikon TMS, DL 40 $\times$ LWD phase-contrast objective). The image was projected with an eyepiece (Nikon HK 15 $\times$) onto a CCD camera (Video Runner 1020, Marshall Electronics) and recorded with a videocassette recorder (Panasonic AG-6300). The time was superimposed onto the video image by a time-date generator (Panasonic WJ-810), and the speed (marker rotation rate) was determined upon video playback from the time interval required for an integral number of revolutions. Voltage clamping and current recording circuitry were provided by a patch clamp (Dagan 3900) controlled by a computer (NEC PowerMate 1.80286, with Index Systems IBX interface and modified C Clamp software). Command voltages are given as the micropipette potential minus the bath potential.

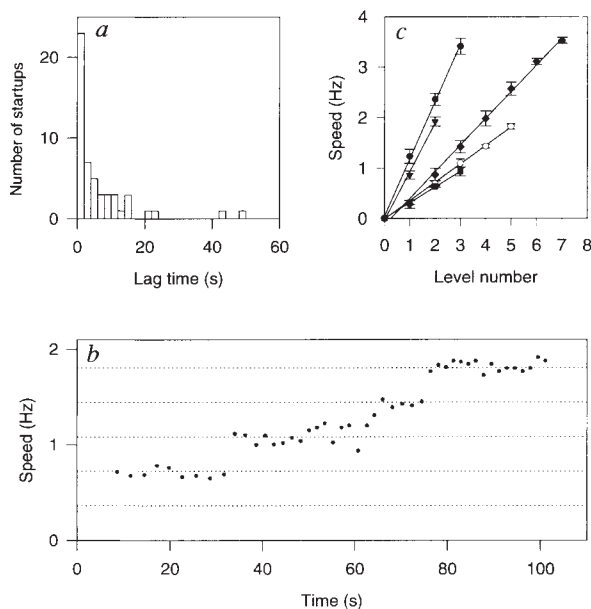


FIG. 2 a, Distribution of lag times for motor startup after the application of a negative command voltage (52 startups with 37 cells). All of the motors were stopped for at least 4 s before a command voltage of either -100 or -150 mV was applied. The distribution was the same for either voltage. The number of motors that remained stopped as a function of time could be fitted by an exponential with a decay time of 8 s (not shown). b, Example of stepwise increase in motor speed following the application of voltage at time 0. This motor started up at level 2. c, Mean speed as a function of level number (determined by inspection) for 5 cells. The data for the cell in b are shown with open symbols. Error bars are standard deviations and solid lines are linear regressions.

to be the motor proteins MotA and/or MotB, which have been shown previously to increment torque in equally sized steps when their expression in paralysed mutants is induced from plasmids^{15,16}. We do not know whether the mechanism of inactivation involves the dissociation of either of these components. The maximum complement of torque generators per motor is thought to be 8 (ref. 16); the largest number of steps seen here was 7. Inactivation and reactivation were noted earlier in the photosynthetic bacterium *Rhodospirillum rubrum*, which stops swimming if incubated anaerobically in the dark: when illuminated, the membrane potential, as monitored by carotenoid bandshifts, recovers much more rapidly than the motility¹⁷. It would be interesting if tethered cells of this species also show stepwise reactivation. The data with positive potentials are consistent with those obtained with artificially energized cell envelopes of *Salmonella typhimurium*, which rotate briefly, if at all, when powered with an outwardly directed proton flux¹⁸. In contrast, cells of *Streptococcus* sustain rotation with a flux of either sign^{5,19,20} but also demonstrate a lag in starting up when initially de-energized⁷.

The dependence of motor speed on command voltage was measured without allowing complete de-energization of cells, in order to maintain a constant number of active torque-generating elements. Only a limited voltage range was accessible: command voltages of greater than about -200 mV destroyed the preparation, presumably because of dielectric breakdown (data not shown). Figure 3a shows time records of command voltage, motor speed and total resistance for three cells. The speed was approximately linear in command voltage (Fig. 3b), reaching a value close to that initially observed when the cell was pulled into the micropipette. However, the speed for a given voltage decreased with time, and the total resistance dropped in a similar fashion. This was attributed to a progressive loss in outer-segment membrane resistance resulting from the diffusion of gramicidin S, either around the cell, through the periplasm, or within

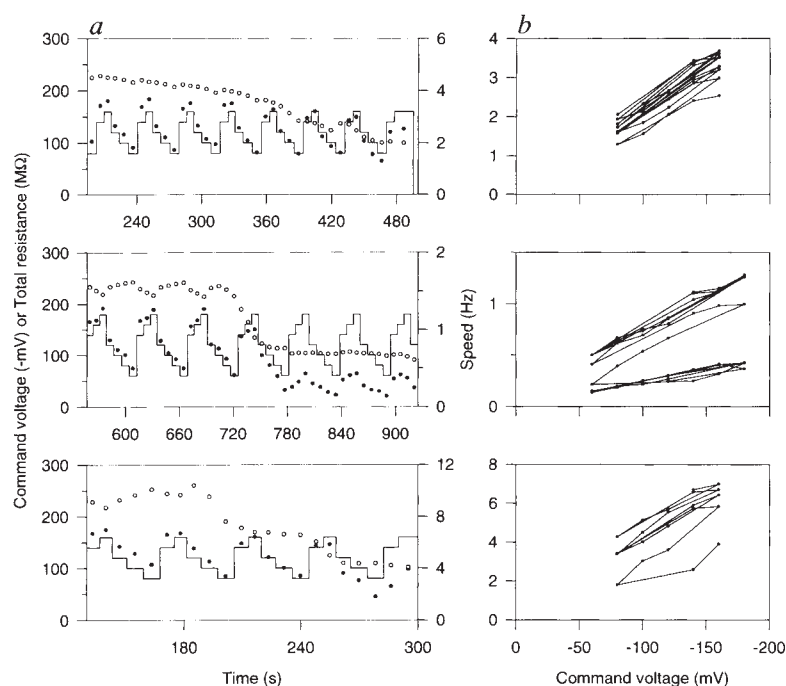
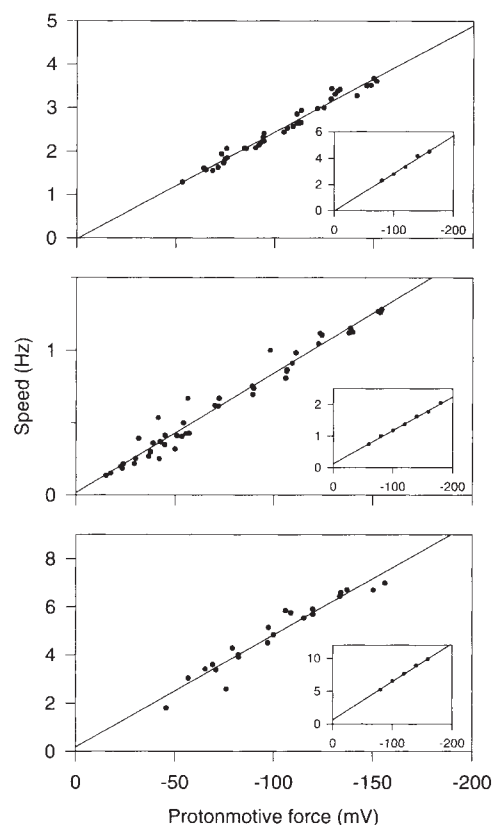


FIG. 3 a, Command voltage (line), motor speed (filled circles) and total resistance (open circles) as a function of time for 3 cells. The time elapsed since the cell was pulled into the micropipette is shown. b, Speed as a function of command voltage for the cells shown in a. Successive voltage steps are joined by lines. The initial speeds of the markers were 3.1, 1.4 and 6.8 Hz, respectively; however, the speed of the first marker already was dropping at the time the video was switched on.

METHODS. The micropipette resistances were 43, 55 and 45 MΩ, respectively. After the cells were drawn into the micropipettes, the total resistances (measured with a 4-mV voltage pulse) were 290, 310 and 260 MΩ, respectively. Then the command voltage was set at -80 mV until a drop in speed was observed, and the voltage-stepping protocol was initiated. The data shown in the figure were collected beginning 1–3 min later. The values shown for the speed and total resistance are the average values for each step. Total resistance = command voltage/total current (Fig. 1d).

FIG. 4 Speed–protonmotive force relation for the cells shown in Fig. 3. Inset, plots validating the assumption that speed is proportional to protonmotive force and the resistance of the inner segment of each cell is approximately constant. The parameter B , defined below, is plotted as a function of V_c . Lines are linear regressions.

METHODS. The decrease in speed over time for fixed command voltage was compensated for as follows. The voltage across the motor (protonmotive force) is given by the voltage-divider equation, $V_m = V_c R_o / (R_i + R_o)$ (see Fig. 1d), assuming that the access resistance of the micropipette is small and the cytoplasm is at a uniform potential. The inner segment of the cell, exposed to gramicidin S inside the micropipette, was well permeabilized when the data were taken, as shown by the initial drop in motor speed and the responsiveness of the motor to changes in command voltage. Thus the decrease in total resistance R_T was due to the gradual diffusion of gramicidin S into the outer segment of the cell causing R_o to fall, with R_i and R_i remaining approximately constant. As shown in Fig. 3, the decrease in R_T is accompanied by a decrease in speed, s . If s varies linearly with V_m , then $s = CV_m$, where C is a constant of proportionality. Defining V_m in terms of the voltage-divider equation and expressing R_o in terms of R_T (the resistance of R_i in parallel with $R_o + R_i$), we obtain $s = CV_c [(R_i + R_i) / R_i] [1 - R_i R_i / (R_i + R_i) R_T]$. This expression is of the form $s = B(1 - A/R_T)$, where $B = CV_c (R_i + R_i) / R_i$ and $A = R_i R_i / (R_i + R_i)$; A is the resistance of R_i in parallel with R_i . For a given cell, several sets of values of s and R_T were obtained for each value of V_c (Fig. 3). For each V_c , these sets were fitted to the equation $s = B(1 - A/R_T)$, using SigmaPlot (Jandel Scientific) to perform the non-linear regressions. The value of A was constrained to be constant for all sets; however, a different value of B was obtained for each value of V_c . B was found to be proportional to V_c (inset), as expected if $B = CV_c (R_i + R_i) / R_i$ and R_i and R_i are constant. The value of A was used to obtain R_i given the further assumption that $R_i \approx (R_T)_{\text{initial}}$. Finally, V_m was calculated from the voltage-divider equation with the knowledge of R_i , R_i and R_T . The plot of s as a function of V_m is shown. The values of A for the three plots were 43, 80 and 62 MΩ, respectively. All of the data are consistent with a linear speed–protonmotive force relation.



the cytoplasmic membrane. It was possible to correct for this loss, as well as for a decrease in total resistance seen at higher command voltages, by assuming that changes in total resistance were due solely to changes in the resistance of the outer-segment membrane, with the leakage and inner-segment membrane resistances remaining constant. We found that speed was proportional to protonmotive force up to at least -150 mV (Fig. 4).

Previous methods for manipulating the protonmotive force used chemical techniques to partially de-energize cells or to gen-

erate diffusion potentials or pH gradients. These methods do not reliably generate values of protonmotive force greater than about -80 mV^{8,9}; however, they have shown that speed is proportional to protonmotive force up to this value^{5–7}. A composite relationship for *Streptococcus* that was approximately linear up to -190 mV has been obtained²¹ by combining data from glycolysing cells, glycolysing cells treated with valinomycin, and starved cells energized by diffusion potentials and/or pH gradients. However, measurements of the protonmotive force of gly-

colysing cells used tetraphosphonium ion, which itself had to be calibrated against diffusion potentials. Large voltages have been applied²² to cells of *Salmonella typhimurium* held by micropipettes, but without dissipation of the endogenous protonmotive force or permeabilization of the cytoplasmic membrane. The data presented here provide direct evidence for individual cells of *E. coli* that the speed of inert markers, and thus motor torque, varies linearly with protonmotive force.

If, on average, each proton that goes through the motor advances the rotor by a fixed angle, then proton flux is proportional to speed. The power input is proton charge $\times$ proton flux $\times$ protonmotive force, and the power output is $2\pi \times$ rotation rate $\times$ torque. Close to stall, where internal energy dissipation tends to zero, the efficiency approaches unity, that is, power

output equals power input²³. In this limit, torque must be proportional to protonmotive force, as observed. Recent evidence indicates that torque for metabolizing *E. coli* is approximately constant over a wide dynamic range, from 0 up to nearly 200 Hz at room temperature²⁴. This led to the suggestion that motor efficiency might be limited by mechanical constraints, that is, torque-generating elements may not stretch (and thus exert forces) beyond a certain point. Were that the case, torque would be expected to level off with protonmotive force. However, our measurements were made at protonmotive forces as high as those expected for metabolizing cells²⁵, so this alternative is untenable. Evidently, diffusion coefficients for mechanical components and rates for proton translocation are such that the motor continues to operate close to equilibrium (at unit efficiency) at relatively high speeds. $\square$

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- Blair, D. F. *Semin. Cell Biol.* **1**, 75–85 (1990).
- Jones, C. J. & Alzawa, S.-I. *Adv. microb. Physiol.* **32**, 109–172 (1991).
- Macnab, R. M. A. *Rev. Genet.* **26**, 131–158 (1992).
- Schuster, S. C. & Khan, S. A. *Rev. Biophys. biomolec. Struct.* **23**, 509–539 (1994).
- Manson, M. D., Tedesco, P. M. & Berg, H. C. *J. molec. Biol.* **138**, 541–561 (1980).
- Conley, M. P. & Berg, H. C. *J. Bact.* **158**, 832–843 (1984).
- Khan, S., Meister, M. & Berg, H. C. *J. molec. Biol.* **184**, 645–656 (1985).
- Meister, M. & Berg, H. C. *Biophys. J.* **52**, 413–419 (1987).
- Meister, M. thesis, California Inst. Technol., Pasadena, CA (1987).
- Katsu, T., Kobayashi, H. & Fujita, Y. *Biochim. biophys. Acta* **860**, 608–619 (1986).
- Ovchinnikov, Y. A. & Ivanov, V. T. in *The Proteins* 3rd edn Vol. 5 (eds Neurath, H. & Hill, R. L.) 307–642 (Academic, New York, 1982).
- Fung, D. C. Y. K. thesis, Harvard Univ., Cambridge, MA (1994).
- Block, S. M., Blair, D. F. & Berg, H. C. *Nature* **338**, 514–517 (1989).
- Block, S. M., Blair, D. F. & Berg, H. C. *Cytometry* **12**, 492–496 (1991).
- Block, S. M. & Berg, H. C. *Nature* **309**, 470–472 (1984).

- Blair, D. F. & Berg, H. C. *Science* **242**, 1678–1681 (1988).
- Armitage, J. P. & Evans, M. C. W. *Biochim. biophys. Acta* **806**, 42–55 (1985).
- Ravid, S. & Eisenbach, M. *J. Bact.* **158**, 222–230 (1984).
- Berg, H. C., Manson, M. D. & Conley, M. P. *Symp. Soc. exp. Biol.* **35**, 1–31 (1982).
- Khan, S. & Berg, H. C. *Cell* **32**, 913–919 (1983).
- Khan, S., Dapice, M. & Humayun, I. *Biophys. J.* **57**, 779–796 (1990).
- Kamiyake, N., Kudo, S. & Hotani, H. *Biophys. J.* **60**, 1350–1355 (1991).
- Meister, M., Lowe, G. & Berg, H. C. *Cell* **49**, 643–650 (1987).
- Berg, H. C. & Turner, L. *Biophys. J.* **65**, 2201–2216 (1993).
- Kashket, E. R. A. *Rev. Microbiol.* **39**, 219–242 (1985).
- Isihara, A., Segall, J. E., Block, S. M. & Berg, H. C. *J. Bact.* **155**, 228–237 (1983).
- Berg, H. C. & Turner, L. *Biophys. J.* **58**, 919–930 (1990).
- Brown, K. T. & Flaming, D. G. *Advanced Micropipette Techniques for Cell Physiology* (Wiley, Chichester, 1986).

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Mechanism of active transcriptional repression by the retinoblastoma protein

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THE retinoblastoma tumour-suppressor protein (Rb) belongs to a family that share a motif known as the pocket. The pocket was originally identified as the region of Rb required for binding to oncoproteins from DNA tumour viruses^{1,2}, which disrupt the binding of Rb to the E2F family of cell-cycle transcription factors (referred to collectively here as E2F)³. Rb switches E2F sites from positive to negative elements⁴, suggesting that Rb–E2F is an active complex that blocks transcription. Here we report that Rb is selectively recruited to promoters through E2F, where it in turn inactivates surrounding transcription factors by blocking their interaction with the basal transcription complex. We suggest that this repressor activity is essential for inhibiting promoters that contain enhancers in addition to E2F sites.

The growth-suppressive domain of Rb was linked to the DNA binding domain of the yeast transcription factor GAL4 (pM2–Rb), so Rb could be brought to a promoter in an E2F-independent fashion (Fig. 1a). To insure that GAL4–Rb has the same activity as wild-type Rb, pM2–Rb was cotransfected with pSVEB–E2F (Fig. 1a). The activity of the simian virus 40 (SV40) enhancer was efficiently blocked by pM2–Rb (Fig. 1b). The E2F sites were then replaced with GAL4 sites (pSVEB–G), and pM2–Rb inhibited the activity of pSVEB–G (Fig. 1a) in a concentra-

tion-dependent fashion (Fig. 1b). These transfections were performed in C33A cells⁵; similar results were obtained in other cell lines and with other enhancer constructs. The Rb pocket (pM2–Rb–P (amino acids 379 to 792)) was sufficient for full repressor activity. Consistent with this result, mutation of the Cys at amino acid 706 to a Phe (pM2–Rb–706), which disrupts pocket function⁶, blocked repressor activity (Fig. 1b). The activity of pSVEB–E2F was not affected by pM2–Rb–P, which was expected because the Rb pocket alone does not bind to E2F efficiently⁷. Western blots show that the fusion proteins are of the predicted size and are expressed at similar levels (Fig. 1c).

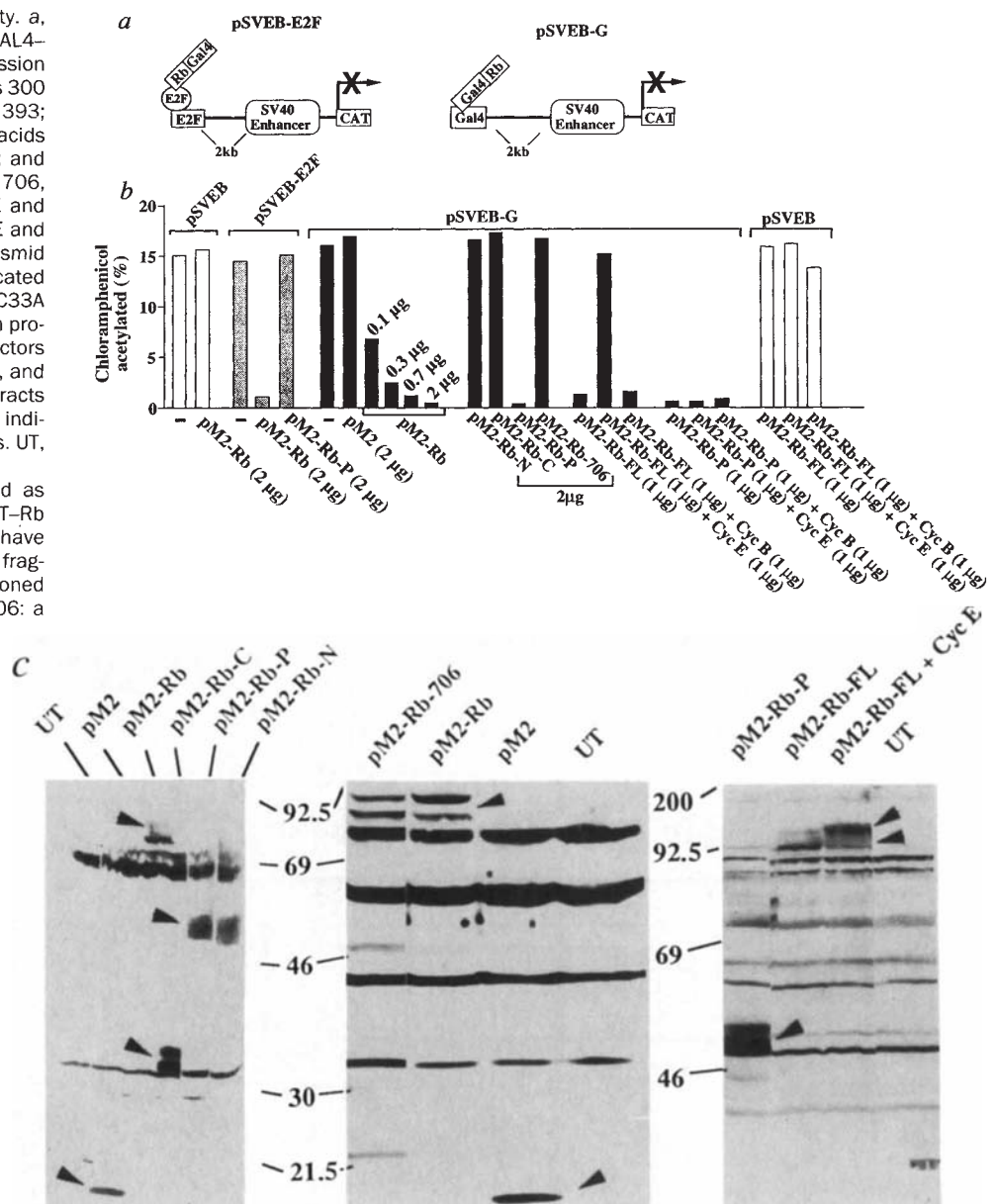
The activity of Rb is controlled by phosphorylation: hyperphosphorylated forms do not bind E2F^{8,9}. An expression vector for cyclin E, which is known to phosphorylate Rb (ref. 10), was cotransfected with GAL4–Rb–FL (amino acids 1 to 928). Full-length Rb was used because amino-terminal deletions inhibit phosphorylation^{7,10}. Overexpression of cyclin E resulted in a shift of GAL4–Rb–FL to a more slowly migrating form in western blots (Fig. 1c), consistent with its phosphorylation^{8,9}, and it blocked repressor activity; cyclin B had no effect (Fig. 1b). Cyclins E and B were expressed in similar levels in the transfected cells, as determined by western blot (results not shown). Repression by pM2–Rb–P (which encodes the small pocket of Rb, amino acids 379 to 792, which is not inactivated by phosphorylation) was unaffected by cyclin E (Fig. 1b). Therefore phosphorylation of Rb not only inhibits binding to E2F, but also disrupts other Rb interactions that are important for general repressor activity.

Even though Rb binds several different transcription factors, experiments to identify promoter elements that are normally targeted by Rb have only identified E2F sites¹¹. Further experiments were designed to test which transcription factors are capable of interacting functionally with Rb at the promoter. Constructs that encode chimaeric proteins in which the GAL4 DNA-binding domain is fused to transcription factors that have been shown to bind to Rb (E2F-1, c-myc, E1f-1 and PU.1)^{8,12–14} were cotransfected with an Rb expression vector. The activity of GAL4–E2F-1 and GAL4–E1a (ref. 15) was completely

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FIG. 1 Rb has intrinsic repressor activity. **a**, Diagram of promoter interactions. **b**, GAL4-Rb is a repressor; pM2 is the GAL4 expression vector; pM2-Rb encodes Rb amino acids 300 to 928 (GAL4-Rb). N, amino acids 1 to 393; P, amino acids 379 to 792; C, amino acids 767 to 928; FL, amino acids 1 to 928; and 706, Cys to Phe mutation at amino acid 706, these all indicate Rb sequences. Cyc E and Cyc B are expression vectors for cyclin E and cyclin B, respectively¹⁰. Reporter plasmid (0.5 µg) was cotransfected with the indicated amount of expression plasmid into C33A cells⁹. **c**, Western blot of GAL4-Rb fusion proteins. The indicated expression vectors (10 µg) were transfected into C33A cells, and western blots were done on protein extracts using anti-GAL4 antiserum. Arrowheads indicate the position of GAL4 fusion proteins. UT, untransfected cells.

METHODS. C33A cells were transfected as described¹⁹. pGT-Rb (379 to 792)²⁰, pGT-Rb (1 to 928)²⁰, and pM2 and pM3 (ref. 22) have all been described. pM2-Rb: an *EcoRI* fragment (amino acids 300 to 928) was cloned into the *EcoRI* site of pM2. pM2-Rb-706: a *Styl* fragment from pRb60 (706) was first cloned into pGEM-1 (Promega) containing full-length human Rb complementary DNA. Then an *EcoRI* fragment from this plasmid was cloned into pM2. pM2-Rb-P: a *BamHI* fragment from pGT-Rb (379 to 792) was cloned into pM2. pM2-Rb-C: *SspI* (amino acids 767)/*SmaI* (vector) fragment from pM2-Rb was cloned into the *SmaI* site of pM3. pM2-Rb-N: the *BamHI* (vector)/*BclI* (amino acid 393) fragment of pGT-Rb (1 to 928) was cloned into the *BamHI* site in pM2. pSVEB: the SV40 enhancer was cloned into the *BglIII* site in pCAT-Promoter (Promega). pSVEB-G: the *PstI/XbaI* fragment containing GAL4 binding sites from pG₅E1bCAT (ref. 15) was cloned into the corresponding sites of pSVEB. pSVEB-E2F: E2F sites from pSKE2F (ref. 4) were cloned into the *PstI/SalI* sites of pSVEB.

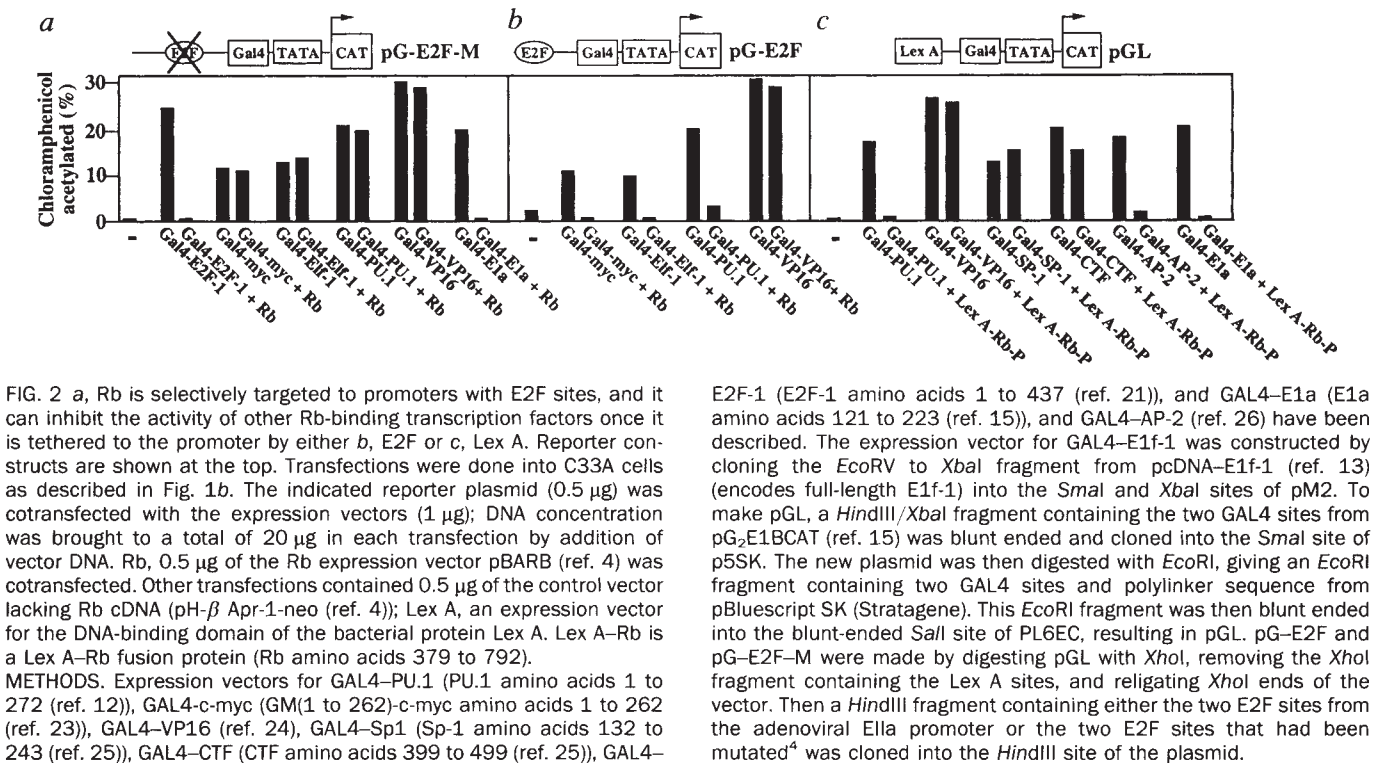


blocked, but there was no effect on the activity of the other fusion proteins (Fig. 2a). We investigated why E2F-1 and E1a are able to recruit Rb to a promoter, whereas the other Rb-binding transcription factors are not. One possibility, supported by *in vitro* binding assays (results not shown), is that the affinity of Rb for E2F-1 and E1a is higher than for the other factors.

Although Rb was unable to block the activity of c-myc, Elf-1 or PU.1 in the experiments described above, if it was tethered to the promoter through an interaction with E2F or artificially through a Lex-A DNA-binding domain, the activity of these proteins was efficiently blocked (Fig. 2b, c). As controls, Rb had little or no effect on the activity of VP16, CTF or Sp1 (Fig. 2b, c), and these proteins did not interact significantly with Rb *in vitro* in binding assays (results not shown), indicating that there is a correlation between binding of Rb and its ability to inhibit transactivating proteins. Together, our results suggest that, when tethered to the promoter through E2F, Rb is functioning by binding to and inactivating adjacent susceptible transcription factors and is not acting as a general inhibitor of the basal transcription complex. If this is the case, Rb should be able to bind simultaneously to E2F and the other susceptible transcription factors. Glutathione *S*-transferase (GST)-E2F-1

fusion protein on glutathione beads was incubated with Rb tagged by His to form an E2F-1-Rb complex. The complex was then incubated with ³⁵S-labelled c-myc, Elf-1 or PU.1. Each of the factors bound specifically to Rb in the E2F-1-Rb complex (Fig. 3a-c), demonstrating that Rb can bind simultaneously to E2F-1 and the other factors. The tags were then switched (that is, His-E2F-1 and GST-Rb), and similar results were obtained. Efficient binding of E2F-1 to DNA requires heterodimerization with DP-1 (ref. 16), and we show that Rb-PU.1 interacts efficiently with the E2F-1/DP-1/DNA complex (Fig. 3d, e). Our results suggest that Rb can interact with E2F-1/DP-1 and other transcription factors when these proteins are bound to DNA.

Several Rb-binding transcription factors have been shown to interact with the TATA-binding protein (TBP), including PU.1, c-myc and E2F-1 (refs 12, 14, 17). TBP exists in a multimeric complex with other factors known as TFIID. Purified TFIID was incubated with GST-PU.1 on glutathione beads, and interaction with TFIID was followed by western blots for two TBP-associated factors, TAF 250 and TAF 55 (ref. 18). To determine whether Rb would block the interaction between PU.1 and TFIID, GST-PU.1 on beads was preincubated with Rb before incubation with TFIID. Rb blocked the interaction of PU.1 with



E2F-1 (E2F-1 amino acids 1 to 437 (ref. 21)), and GAL4-E1a (E1a amino acids 121 to 223 (ref. 15)), and GAL4-AP-2 (ref. 26) have been described. The expression vector for GAL4-E1f-1 was constructed by cloning the EcoRV to XbaI fragment from pcDNA-E1f-1 (ref. 13) (encodes full-length E1f-1) into the SmaI and XbaI sites of pM2. To make pGL, a HindIII/XbaI fragment containing the two GAL4 sites from pG₂E1BCAT (ref. 15) was blunt ended and cloned into the SmaI site of p5SK. The new plasmid was then digested with EcoRI, giving an EcoRI fragment containing two GAL4 sites and polylinker sequence from pBluescript SK (Stratagene). This EcoRI fragment was then blunt ended into the blunt-ended SmaI site of PL6EC, resulting in pGL. pG-E2F and pG-E2F-M were made by digesting pGL with XhoI, removing the XhoI fragment containing the Lex A sites, and religating XhoI ends of the vector. Then a HindIII fragment containing either the two E2F sites from the adenoviral E1a promoter or the two E2F sites that had been mutated⁴ was cloned into the HindIII site of the plasmid.

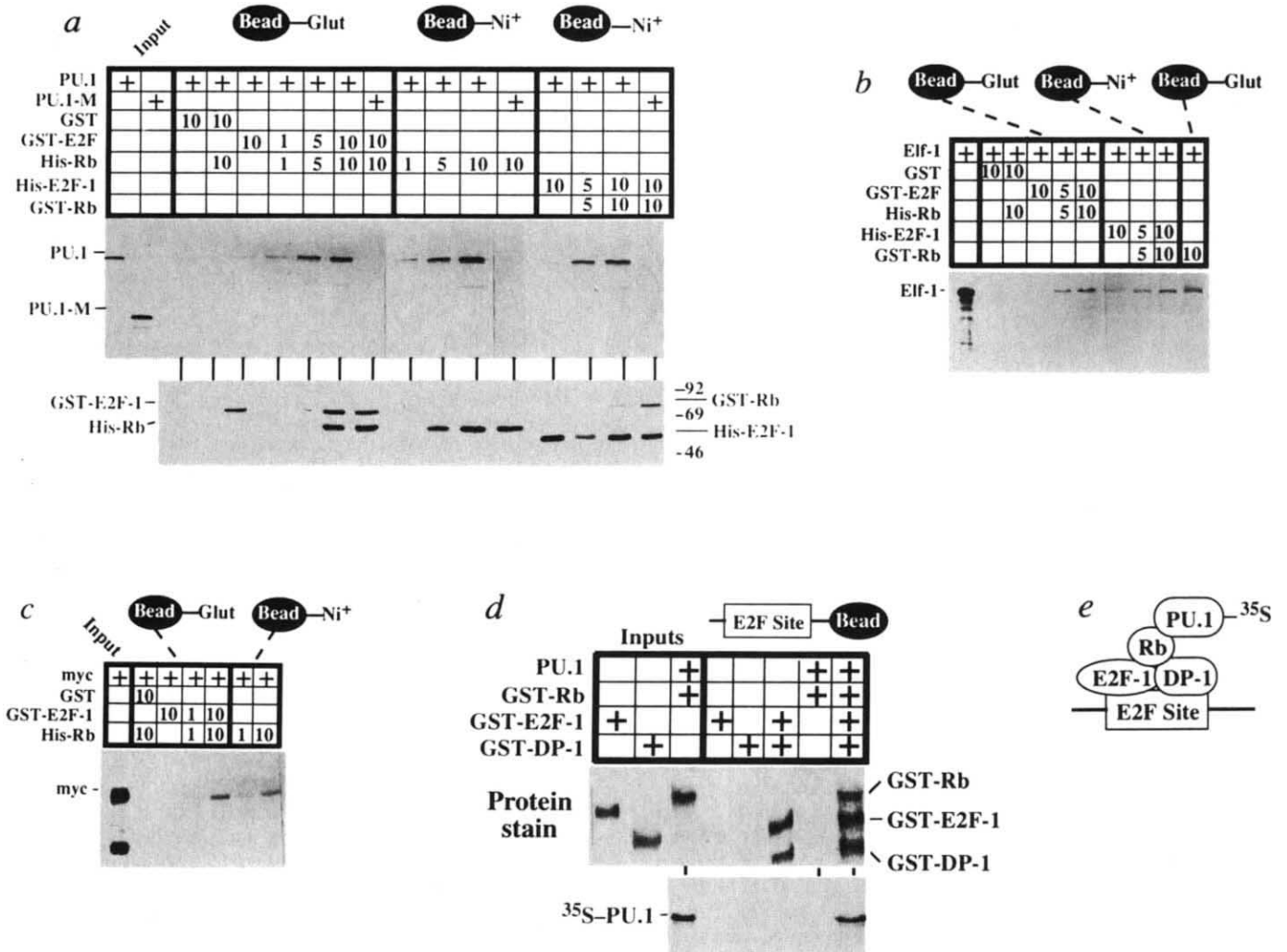
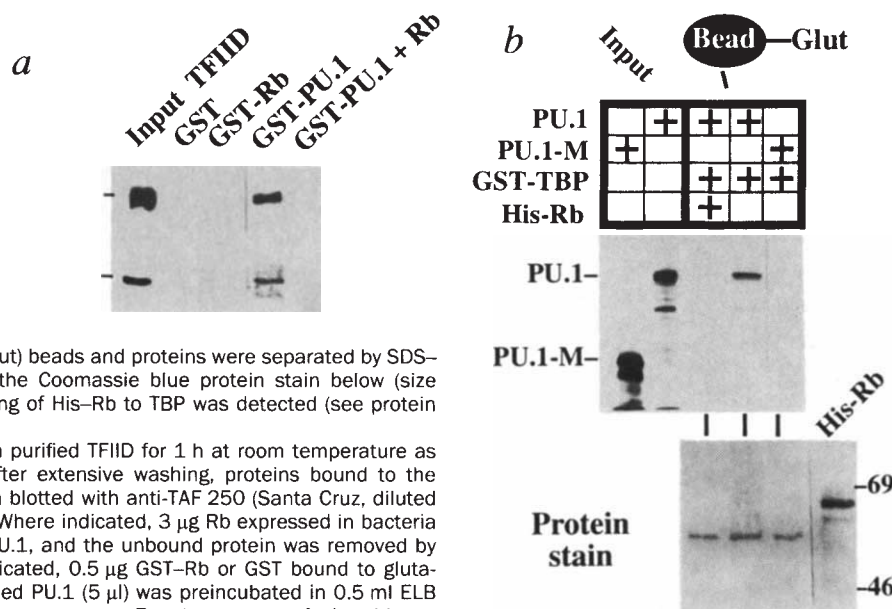


FIG. 4 Rb blocks the interaction of PU.1 with TFIID and TPB. **a**, Rb blocks binding of PU.1 to TFIID. Purified TFIID was incubated with GST-PU.1 on glutathione Sepharose beads, and the interaction of TFIID was followed by western blots for TAF 250 and TAF 55, which are indicative of TFIID. Where indicated, Rb was incubated with GST-PU.1, and unbound Rb was washed away before incubation with TFIID. GST-Rb or GST alone on glutathione Sepharose beads did not interact with TFIID. **b**, Rb blocks interaction of PU.1 with TBP. 35 S-labelled PU.1 or PU.1-M was incubated with GST-TBP in the presence or absence of His-Rb. Complexes were isolated using glutathione (Glut) beads and proteins were separated by SDS-PAGE. The autoradiogram is shown above and the Coomassie blue protein stain below (size markers show relative molecular mass). No binding of His-Rb to TBP was detected (see protein stain).

METHODS. GST-PU.1 (0.5 μ g) was incubated with purified TFIID for 1 h at room temperature as described¹⁸ in EBC containing 250 mM NaCl. After extensive washing, proteins bound to the beads were separated by SDS-PAGE and western blotted with anti-TAF 250 (Santa Cruz, diluted 1:100) or anti-TAF-55 (ref. 28; diluted 1:3,000). Where indicated, 3 μ g Rb expressed in bacteria or in baculovirus²⁹ was preincubated with GST-PU.1, and the unbound protein was removed by washing before incubation with TFIID. Where indicated, 0.5 μ g GST-Rb or GST bound to glutathione beads was incubated with TFIID. 35 S-labelled PU.1 (5 μ l) was preincubated in 0.5 ml ELB (300 mM) with eluted His-Rb for 1 h at room temperature. Equal amounts of glutathione-Sepharose bound GST-TBP were then added to this binding reaction mixture and to 0.5 ml of ELB (300 mM NaCl) that contained 5 μ l 35 S-PU.1. These reactions were incubated with gentle agitation for 1 h at room temperature, and the Sepharose beads were then washed extensively with ELB and analysed by SDS-PAGE and Coomassie blue staining and autoradiography. All other binding assays were performed and analysed as in Fig. 3, except that the Sepharose-bound GST fusion proteins were purified and then placed directly into 0.5 ml EBC with albumin (1 mg ml⁻¹) along with 35 S-PU.1 without the preincubation steps, and after the incubation period the beads were washed with NETN.



TFIID (Fig. 4a). No interaction of Rb with TFIID was detected, suggesting that the binding of Rb to PU.1 blocks the interaction of PU.1 with TFIID. The interaction of PU.1 with purified TBP was also blocked by Rb (Fig. 4b), suggesting that PU.1 may interact with TFIID through TBP. We investigated how Rb blocks the interaction of transcription factors with TBP. The Rb pocket shows sequence similarity to TBP and TFIIB (ref. 12), suggesting that it could mimic components of the basal transcription complex and sequester susceptible transcription factors in inactive complexes.

We suggest that Rb is selectively targeted to promoters through interactions with E2F. Once tethered through E2F, Rb binds to adjacent, susceptible transcription factors, blocking

their interaction with the basal transcription complex. This repressor activity appears crucial for inhibiting the activity of promoters that have enhancers in addition to E2F sites. □

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- Hu, Q. J., Dyson, N. & Harlow, E. *EMBO J.* **9**, 1147-1155 (1990).
- Kaelin, W. Jr, Ewen, M. E. & Livingston, D. M. *Molec. cell. Biol.* **10**, 3761-3769 (1990).
- Bagchi, S., Raychaudhuri, P. & Nevins, J. R. *Cell* **62**, 659-669 (1990).
- Weintraub, S. J., Prater, C. A. & Dean, D. C. *Nature* **358**, 259-261 (1992).
- Scheffner, M., Munger, K., Byrne, J. C. & Howley, P. M. *Proc. natn. Acad. Sci. U.S.A.* **88**, 5523-5527 (1991).
- Kaye, F. J., Kratzke, R. A., Gerster, J. L. & Horowitz, J. M. *Proc. natn. Acad. Sci. U.S.A.* **87**, 6922-6926 (1990).
- Qian, Y., Luckey, C., Horton, L., Esser, M. & Templeton, D. J. *Molec. cell. Biol.* **12**, 5363-5372 (1992).
- Chen, P. L., Scully, P., Shew, J. Y., Wang, J. Y. & Lee, W. H. *Cell* **58**, 1193-1198 (1989).
- DeCaprio, J. A. et al. *Cell* **58**, 1085-1095 (1989).
- Hinds, P. W. et al. *Cell* **70**, 993-1006 (1992).
- Chittenden, T., Livingston, D. M. & Kaelin, W. Jr *Cell* **65**, 1073-1082 (1991).
- Hagemeier, C., Bannister, A. J., Cook, A. & Kouzarides, T. *Proc. natn. Acad. Sci. U.S.A.* **90**, 1580-1584 (1993).
- Wang, C. Y., Petryniak, B., Thompson, C. B., Kaelin, W. G. & Leiden, J. M. *Science* **260**, 1330-1335 (1993).
- Hateboer, G. et al. *Proc. natn. Acad. Sci. U.S.A.* **90**, 8489-8493 (1993).
- Martin, K. J., Lillie, J. W. & Green, M. R. *Nature* **346**, 147-152 (1990).
- Girling, R. et al. *Nature* **362**, 83-87 (1993).
- Hagemeier, C., Cook, A. & Kouzarides, T. *Nucleic Acids Res.* **21**, 4998-5004 (1993).
- Kashanchi, F. et al. *Nature* **367**, 295-299 (1994).
- Iademarco, M. F., McQuillan, J. J., Rosen, G. D. & Dean, D. C. *J. biol. Chem.* **267**, 16323-16329 (1992).
- Kaelin, W. Jr, Pallas, D. C., DeCaprio, J. A., Kaye, F. J. & Livingston, D. M. *Cell* **64**, 521-532 (1991).
- Helin, K. et al. *Genes Dev.* **7**, 1850-1861 (1993).
- Sadowski, I., Bell, B., Broad, P. & Hollis, M. *Gene* **118**, 137-141 (1992).
- Kato, G. J., Barrett, J., Villa-Garcia, M. & Dang, C. V. *Molec. cell. Biol.* **10**, 5914-5920 (1990).
- Sadowski, I., Ma, J., Triezenberg, S. & Ptashne, M. *Nature* **335**, 563-564 (1988).
- Seipel, K., Georgiev, O. & Schaffner, W. *EMBO J.* **11**, 4961-4968 (1992).
- Tanese, N., Pugh, B. F. & Tjian, R. *Genes Dev.* **5**, 2212-2224 (1991).
- Smith, D. B. & Johnson, K. S. *Gene* **67**, 31-40 (1988).
- Chiang, C.-M. & Roeder, R. G. *Science* **267**, 531-536 (1995).
- Dowdy, S. F. et al. *Cell* **73**, 499-511 (1993).

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◀ FIG. 3 Rb can bind simultaneously to E2F-1 and other transcription factors. **a**, GST-E2F-1 on glutathione (Glut) Sepharose beads was incubated with an excess of His-Rb to perform an E2F-Rb complex. E2F-Rb was then incubated with 35 S-labelled PU.1 or PU.1-M (non-binding mutant)¹². Autoradiograms are shown at the top, with the Coomassie blue protein stain below. For E2F-1 and GST, 10 indicates 2 μ g. **b**, Binding of 35 S-E1f-1 to E2F-1-Rb. **c**, Binding of 35 S-c-myc to E2F-1-Rb. **d**, PU.1 binds Rb in the Rb/E2F-1/DP-1 complex bound to DNA. A 35 S-PU.1/GST-Rb complex was preformed on glutathione beads, the complex was eluted and then allowed to interact with an E2F-1/DP-1 heterodimer bound to a biotin-tagged E2F site attached to avidin sepharose beads. **e**, Interactions in **d**. **METHODS.** Expression vectors and GST protein isolation have been described^{12-14,20,21,27}. After binding to glutathione-Sepharose (Pharmacia), proteins were washed extensively with ELB (ref. 21). Proteins were eluted with 10 mM reduced glutathione (Sigma) in ELB. His proteins were purified as recommended by the manufacturer (Novagen). 35 S-labelled proteins were made using the TNT Coupled Transcription/Translation System (Promega). Binding of complexes to 35 S-proteins was for 1 h at room temperature; beads were then washed with NETN (ref. 20). Ni-Sepharose beads were washed with 100 mM imidazole in NETN. GST-E2F-1 and GST-DP-1 (ref. 16) were incubated with biotin-tagged E2F sites⁴ bound to avidin Sepharose beads. 35 S-PU.1 was incubated with GST-Rb on glutathione Sepharose beads, and the complex was then eluted with glutathione. 35 S-PU.1/GST-Rb was then incubated with the E2F-1/DP-1/DNA complex in 20 mM HEPES (pH 7.6), 10% glycerol, 100 mM KCl, 1 mM MgCl₂, and 0.1 mM NP-40, and then washed extensively with the same buffer containing 100 mM NaCl.